PCT/AU2004/001570

Australian Government

Patent Office
Canberra

I, JANENE PEISKER, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003906261 for a patent by THE UNIVERSITY OF SYDNEY as filed on 13 November 2003.

COMMONWEALTH OF AUSTRALIA
AUSTRALIA
PATENT OFFICE

WITNESS my hand this
First day of February 2005

JANENE PEISKER
TEAM LEADER EXAMINATION
SUPPORT AND SALES

BEST AVAILABLE COPY

S&F Ref: 654008

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION FOR THE INVENTION ENTITLED:

Bis-cationic Compounds and Use Thereof

Name and Address of Applicant:

The University of Sydney, a body corporate established pursuant to the University of Sydney Act 1989 (NSW), of Parramatta Road, Sydney, New South Wales, 2006, Australia

Names of Inventors:

Tania Sorrell and Lesley Wright and Alfred Widmer and Katrina Jolliffe

This invention is best described in the following statement:

Bis-cationic Compounds and Use Thereof

Technical Field

The present invention relates to bis-cationic compounds comprising quaternary ammonium groups and/or quaternary phosphonium groups. The invention also relates to the use of bis-cationic compounds as Phospholipase B inhibitors and in the treatment or prevention of microbial infection.

Background of the Invention

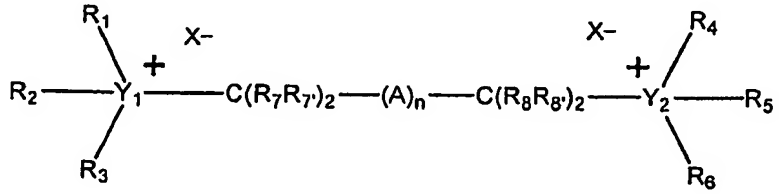
There is a need in therapy and industry for improved antimicrobial agents. The phospholipase(s) have attributes of virulence factors and thus, may be a useful target for at least one of treatment, inhibition and prevention of microbial infection, such as fungal, bacterial, viral or parasitic, infection.

Hanel et al (*Mycosis*, 1995, 38:252-264) investigated the role of fungal phospholipases as drug targets in a mouse model of *Candida albicans* infection. Mice were treated with beta blockers and related compounds which inhibited secretory phospholipase activity on egg yolk plate assays. However, as discussed by Ashraf et al. Microbiology (1997), 143, 331-340, the assays used by Hanel et al are not specific for phospholipases.

The present invention is directed to bis-cationic compounds comprising quaternary ammonium groups and/or quaternary phosphonium groups and their potential use as inhibitors of phospholipases, including intracellular and secretory phospholipases, for treating, inhibiting, or preventing microbial infection.

Summary of the Invention

According to a first aspect of the invention there is provided a compound of formula (I)



(I)

wherein

Y_1 and Y_2 may be the same or different and are selected from N and P;

X- is a counterion;

R_1 to R_6 may be the same or different and are independently selected from the group consisting of optionally substituted C_{1-10} alkyl, optionally substituted C_{2-10} alkenyl, optionally substituted C_{2-10} alkynyl, optionally substituted C_{3-10} cycloalkyl, optionally substituted aryl and optionally substituted aralkyl, wherein said substituents are independently selected from C_{1-3} alkyl, hydroxyl and halogen; or

R_1 and R_2 together with the Y_1 group to which they are attached may optionally form an heterocycloalkyl group; and R_4 and R_5 together with the Y_2 group to which they are attached may optionally form a heterocycloalkyl group; wherein said heterocycloalkyl group may be optionally substituted with one or more groups selected from C_{1-3} alkyl, hydroxyl and halogen;

each A is independently selected from CR_9R_{9a}, optionally substituted phenyl, and optionally substituted C_{5-7} cycloalkyl, wherein said substituents are independently selected from C_{1-3} alkyl, hydroxyl and halogen;

n is an integer from 5 to 18 or optionally 7 to 16;

R_7, $R_{7'}$, R_8 and $R_{8'}$ may be the same or different and are independently selected from hydrogen, F and Cl;

R_9 and R_{9a} may be the same or different and are independently selected from the group consisting of hydrogen, halogen, COR_{10}, OR_{11}, CH_2OR_{11}, CH_2OH, SR_{11}, $NR_{12}R_{13}$, $CON_{12}R_{13}$, OH, SH, amino acids, oligoamino acids, dipeptidyl, tripeptidyl, tetrapeptidyl and pentapeptidyl;

R_{10} is OH, OR_{11}, optionally substituted amino-C_{1-6}-alkylsulfonate, optionally substituted amino-C_{1-6}-alkylphophonate, optionally substituted amino-C_{1-6}-alkyl-guanidinyl, and optionally substituted amino-C_{1-6}-alkyl-tri(C_{1-6}-alkyl)ammonium;

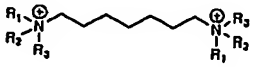
R_{11} is selected from the group consisting of optionally substituted C_{1-10} alkyl, optionally substituted C_{2-10} alkenyl, optionally substituted C_{2-10} alkynyl, optionally substituted C_{3-10} cycloalkyl, and optionally substituted aralkyl, optionally substituted amino-C_{1-6}-alkylsulfonate, optionally substituted amino-C_{1-6}-alkylphophonate, optionally substituted amino-C_{1-6}-alkyl-guanidinyl, and optionally substituted amino-C_{1-6}-alkyl-tri(C_{1-6}-alkyl)ammonium, wherein said optional substituents are independently selected from C_{1-3} alkyl, hydroxyl and halogen;

R_{12} and R_{13} are independently selected from the group consisting of hydrogen, optionally substituted C_{1-10} alkyl, optionally substituted C_{2-10} alkenyl, optionally substituted C_{2-10} alkynyl, optionally optionally substituted C_{3-10} cycloalkyl, optionally

substituted amino-C_{1-6}-alkylsulfonate, optionally substituted amino-C_{1-6}-alkylphophonate, optionally substituted amino-C_{1-6}-alkyl-guanidinyl, and optionally substituted amino-C_{1-6}-alkyl-tri(C_{1-6}-alkyl)ammonium, wherein said substituents are independently selected from C_{1-3} alkyl, hydroxyl, halogen, amino, and $C(O)OR_{11}$; or R_{12} and R_{13} together with the N to which they are attached may optionally form a heterocycloalkyl group; and

R_{12} and R_{13}, together with the nitrogen atom to which they are attached may optionally form an optionally substituted heterocycloalkyl or heteroaryl ring, wherein said substituents are independently selected from C_{1-3} alkyl, hydroxyl, halogen, amino, and $C(O)OR_{11}$;

provided that the compound of formula (I) is not selected from the following:



(C7 linker)

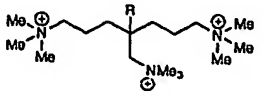
R1 = R2= R3 = Me, Et, 

R1 = R2 = Me, R3 = Et, Pr

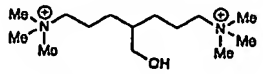
R1 = R2 = Et, R3 = Me

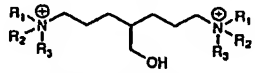
R1 = R2 = Pr, R3 = Me

R1 = R2 = allyl, R3 = Me

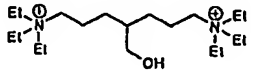


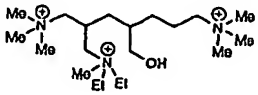
R = NO_2, NH_2

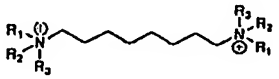




R1= R2 = Me, R3 = Pr
R1=R2=Pr, R3 = Me







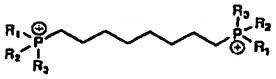
(C8 linker)

R1 = R2 = R3 = Me, Et, Pr, Bu, pentyl, allyl

R1 = R2 = Me, R3 = Pr, Bu, Decyl

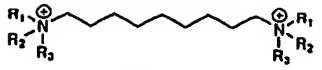
R1 = Me, R2 = R3 = Hexyl, allyl

R1 = Me, R2 = Bu, R3 = octyl



(C8 linker)

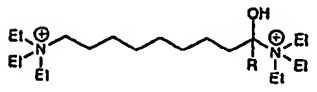
R1 = R2 = R3 = n-Bu, t-Bu, octyl



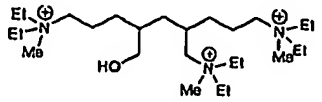
R1 = R2= R3 = Me, Et, allyl

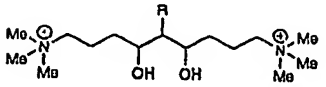
R1 = R2 = Me, R3 = Pr, pentyl

R1 = R2 = allyl, R3 = Et

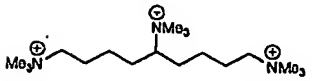


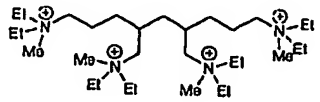
R = heptyl, nonyl, octyl

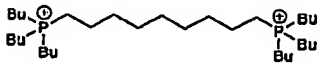


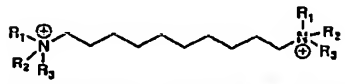


R = Pr, H, pentyl, hexyl, butyl, Me, Et







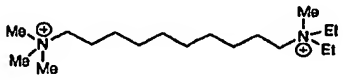


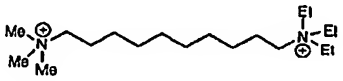
R1 = R2 = R3 = Me, Pr, pentyl, butyl, allyl, ethyl, hexyl

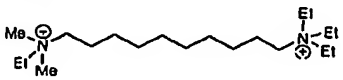
R1 = R2 = Me, R3 = Bu, Et, hexyl, heptyl, pentyl, propyl, decyl, i-Pr, octyl

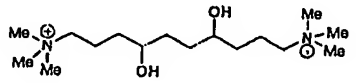
R1 = Me, R2 = R3 = allyl, ethyl

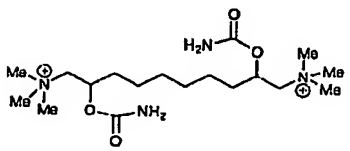
R1 = R2 = Et, R3 = hexyl

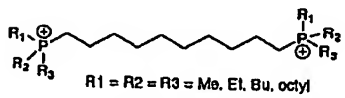




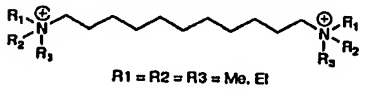






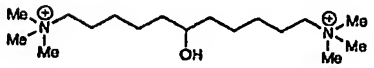


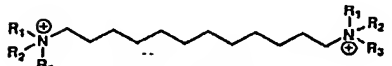
R1 = R2 = R3 = Me, Et, Bu, octyl



R1 = R2 = R3 = Me, Et

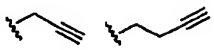
R1 = R2 = Me, R3 = pentyl





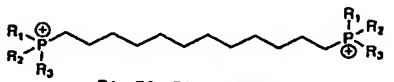
R1 = R2 = R3 = Bu, Et, Pr

R1 = R2 = Me, R3 = Bu, Et, heptyl, nonyl,



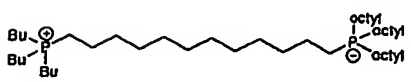
R1 = R2 = allyl, R3 = Me, Et

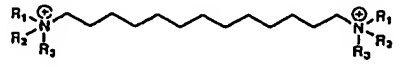
R1 = R2 = hexyl, R3 = Me



R1 = R2 = R3 = octyl, butyl

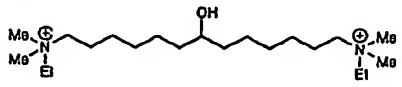
(C12 linker)

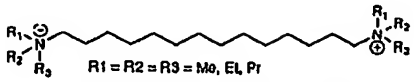




R1 = R2 = R3 = Me, Et

(C13 linker)

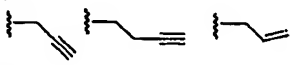




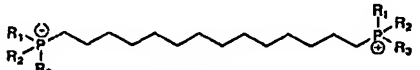
(C14 linker)

R1 = R2 = R3 = Me, Et, Pr

R1 = R2 = Me, R3 =

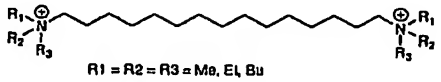


R1 = Me, R2 = Et, R3 = Pr



(C14 linker)

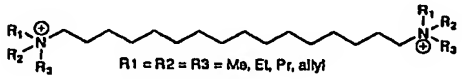
R1 = R2 = R3 = Et



(C15 linker)

R1 = R2 = R3 = Me, Et, Bu

R1 = Me, R2 = Et, R3 = Pr



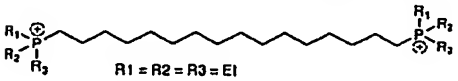
(C16 linker)

R1 = R2 = R3 = Me, Et, Pr, allyl

R1 = R2 = Me, R3 = Et

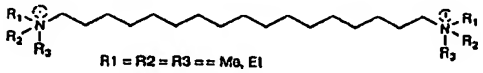
R1 = R2 = Et, R3 = Me

R1 = Me, R2 = Et, R3 = Pr



(C16 linker)

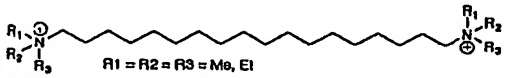
R1 = R2 = R3 = Et



(C17 linker)

R1 = R2 = R3 = = Me, Et

R1 = Me, R2 = Et, R3 = Pr

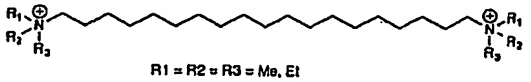


(C18 linker)

R1 = R2 = R3 = Me, Et

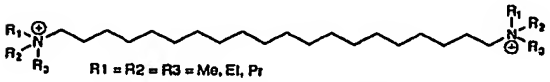
R1 = R2 = Et, R3 = Me

R1 = Me, R2 = Et, R3 = Pr



(C19 linker)

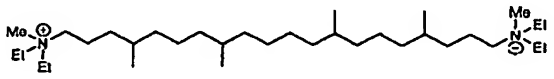
R1 = R2 = R3 = Me, Et

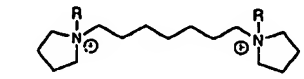


(C20 linker)

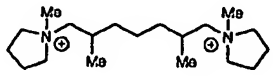
R1 = R2 = R3 = Me, Et, Pr

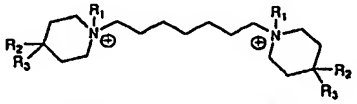
R1 = Me, R2 = Et, R3 = Pr



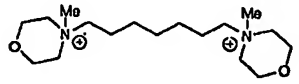


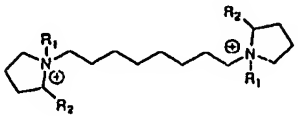
R = Me, Bu



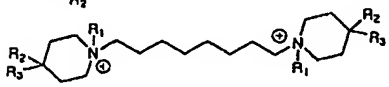


R1 = Me; R2, R3 = H
R1 = Me, R2 = Ph, R3 = CO_2Et



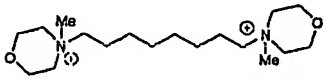


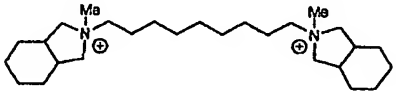
R1 = Me, R2 = H
R1 = Me, R2 = Me
R1 = Me, R2 = Et

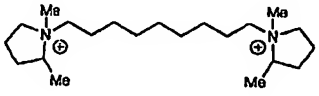


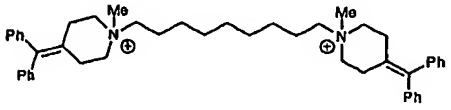
R1 = Me, R2 = Ph, R3 = CO_2Et

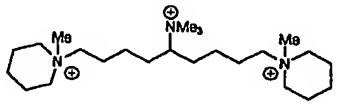
R1 = Me, R2, R3 = 

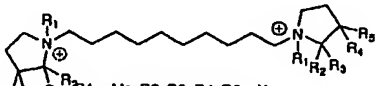




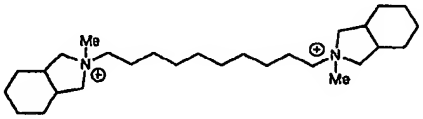


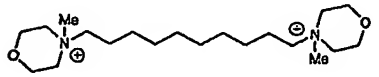


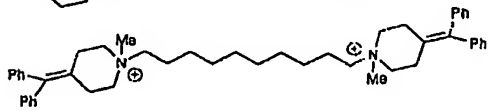


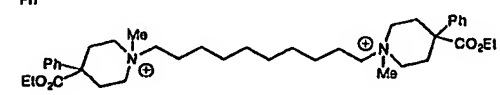


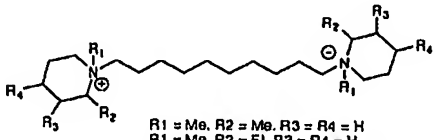
R1 = Me, R2, R3, R4, R5 = H
R1 = Me, R2 = Me, R3, R4, R5 = H
R1 = R2 = R3 = R4 = R5 = Me



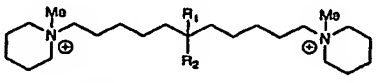




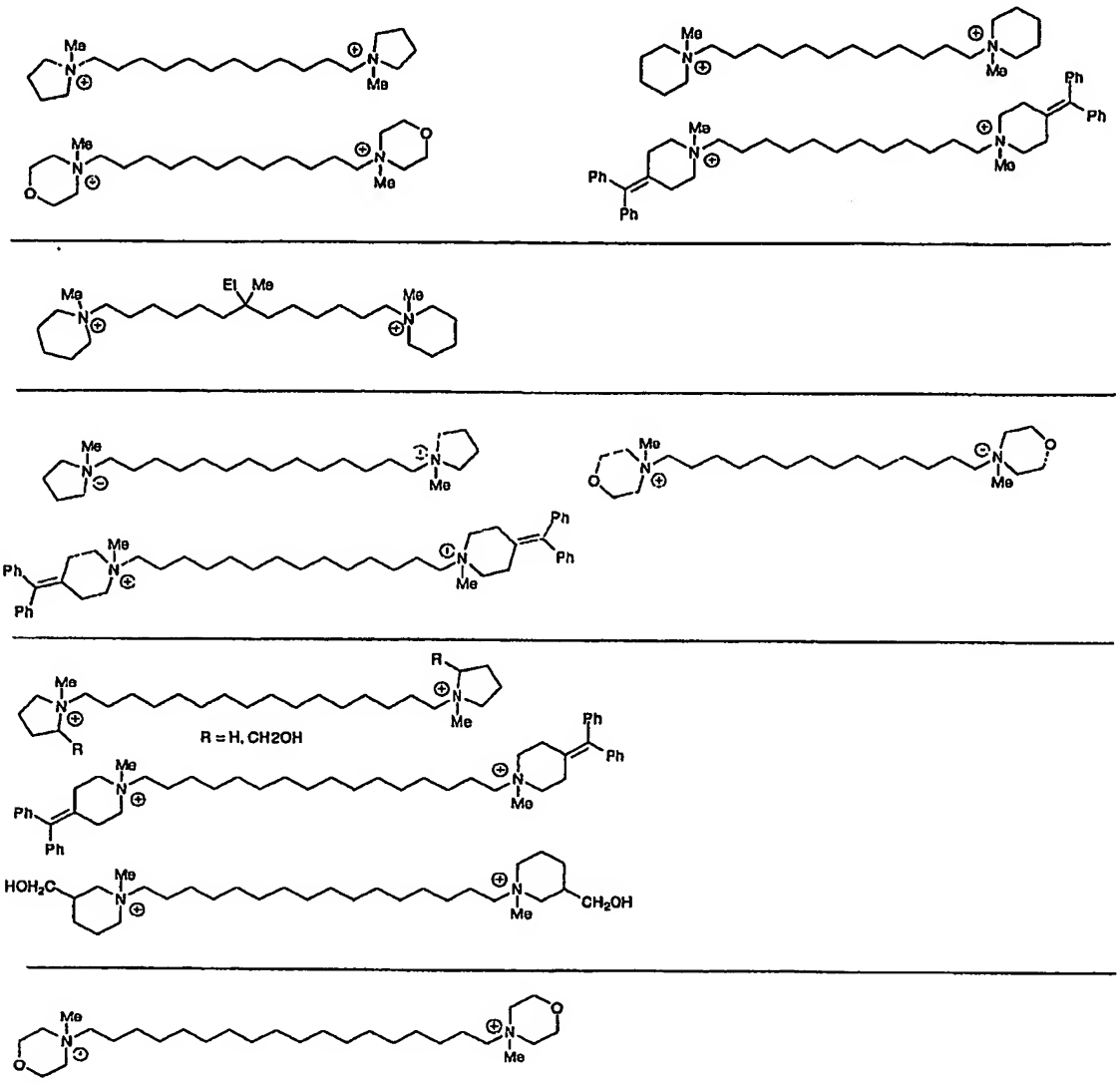




R1 = Me, R2 = Me, R3 = R4 = H
R1 = Me, R2 = Et, R3 = R4 = H
R1 = Et, R2 = H, R3 = OH, R4 = H
R1 = Me, R2 = R3 = R4 = H
R1 = Me, R2 = R3 = R4 = H
R1 = Me, R2 = H, R3 = OC(=O)Pr, R4 = H
R1 = Me, R2 = H, R3 = OAc, R4 = H
R1 = Me, R2 = R3 = H, R4 = OC(O)Ph



R1, r2 = H
R1 = Me, R2 = Et



In one embodiment of the invention Y_1 is N and Y_2 is N. In another embodiment of the invention, Y_1 is N and Y_2 is P. In a further embodiment of the invention Y_1 is P and Y_2 is P.

In accordance with the present ~~invention~~ X^- may be any suitable counterion, including for example, a halide (such as Cl^-, Br^-, I^-), carboxylates, citrate, acetate, $CF_3CO_2^-$, tosylate, BF_4^-, PF_6^-, and OH^-. The counterion may be varied using techniques known to those skilled in the art, for example, ion exchange columns and crystallisation.

In one embodiment of the invention R_1 to R_6 may be the same or different and are independently selected from C_{3-8} alkyl. In another embodiment, R_1 to R_6 may be the same or different and are independently selected from C_{3-6} alkyl.

In some embodiments of the invention, the relative lengths of R_1, R_2 and R_3 may vary by 2 to 6 carbons, for example, 2, 3, 4, 5 or 6 carbons. In other embodiments of the invention, the relative lengths of R_1, R_2 and R_3 may vary by 3 or 4 carbons.

In some embodiments of the invention, the relative lengths of R_4, R_5 and R_6 may vary by 2 to 6 carbons, for example, 2, 3, 4, 5 or 6 carbons. In other embodiments of the invention, the relative lengths of R_4, R_5 and R_6 may vary by 3 or 4 carbons.

In accordance with the first aspect of the invention, n is an integer selected from 5 to 18. For example, n may be 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18.

In one embodiment of the invention, each A is independently selected from CR_9R_{9a}, optionally substituted phenyl, and optionally substituted C_{5-6} cycloalkyl, wherein said substituents are independently selected from C_{1-3} alkyl, hydroxyl and halogen. In one embodiment of the invention, at least one A is CR_9R_{9a}. In one embodiment of the invention, at least one A is phenyl. In another embodiment of the invention, at least one A is cyclohexyl.

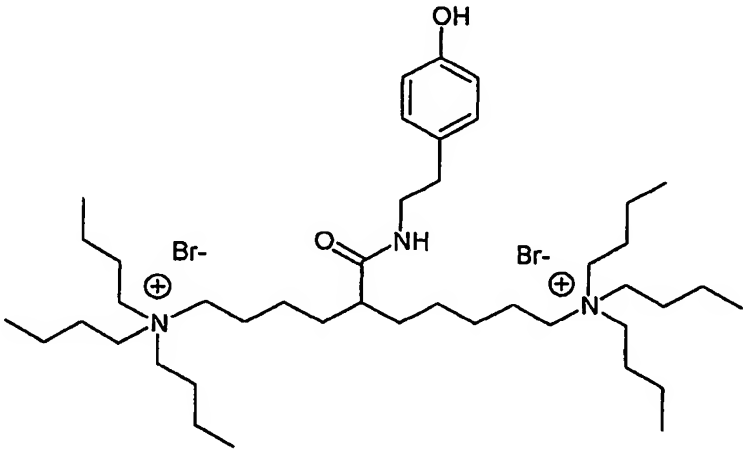
In one embodiment of the invention, R_9 and R_{9a} are both hydrogen. In another embodiment, R_9 is hydrogen and R_{9a} is selected from hydrogen, COR_{10}, OR_{11}, $CONR_{11}R_{12}$, $NR_{12}R_{13}$, OH and SH. For example, in one embodiment of the invention, R_9 is hydrogen and R_{9a} is hydrogen, COOH, CO_2Me, CO_2Et, CO_2Bu, CONHMe, $CONMe_2$, CONHEt, $CONEt_2$, $CONH(CH_2Ph)$, $CON(CH_2Ph)_2$, CO_2Bu^t, OMe, NMe_2, or NEt_2.

With reference to R_{10}, R_{11}, R_{12} and R_{13}, amino-C_{1-6}-alkylsulfonate includes for example, aminomethyl- aminoethyl- and aminopropyl-sulfonate; amino-C_{1-6}-alkylphosphonate includes for example, aminomethyl-, aminoethyl- and aminopropyl-phosphonate; amino-C_{1-6}-alkyl-guanidinyl includes for example, 4-aminobutyl-guanidinyl; amino-C_{1-6}-alkyl-tri(C_{1-6}-alkyl)ammonium includes for example 1-(3-aminopropyl)trimethylammonium.

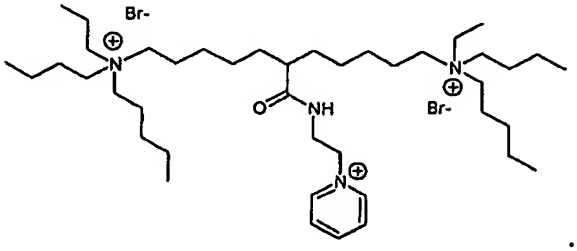
In some embodiments, R_{12} and R_{13}, together with the nitrogen atom to which they are attached may form an optionally substituted heterocycloalkyl or heteroaryl ring. For example, the group $NR_{12}R_{13}$ may optionally be 3-aminopyrrolidinyl, 3-aminoquinalidinyl, 1-(3-aminopropyl)-2-pipecolinyl groups.

In one embodiment of the invention, n is 8, 9 or 10, each A is CR_9R_{9a}, and R_9 and R_{9a} are each hydrogen.

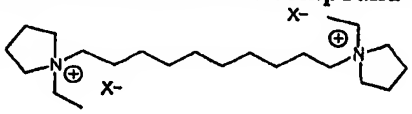
In another embodiment of the invention the compound of formula (I) is:



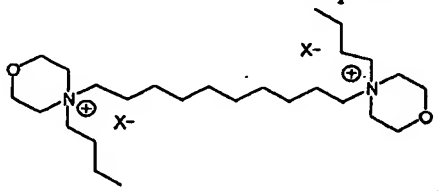
In another embodiment of the invention the compound of formula (I) is:



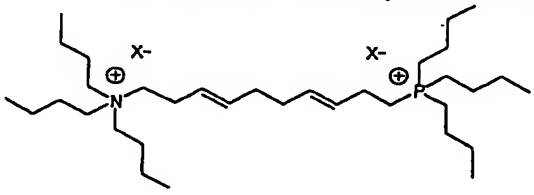
In another embodiment of the invention the compound of formula (I) is:



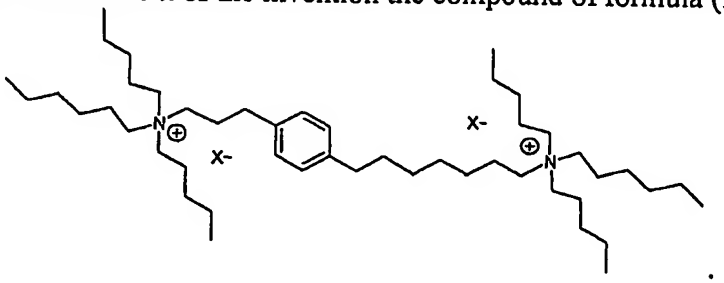
In another embodiment of the invention the compound of formula (I) is:



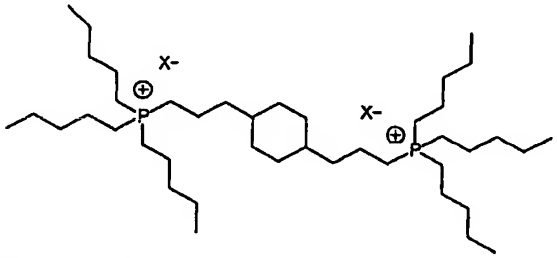
In another embodiment of the invention the compound of formula (I) is:



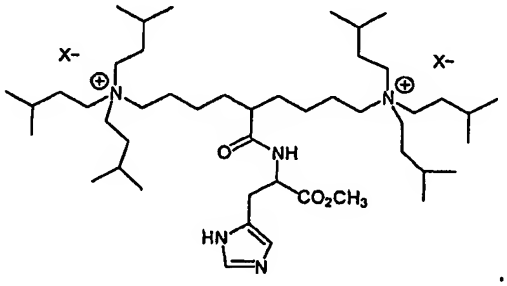
In a further embodiment of the invention the compound of formula (I) is:



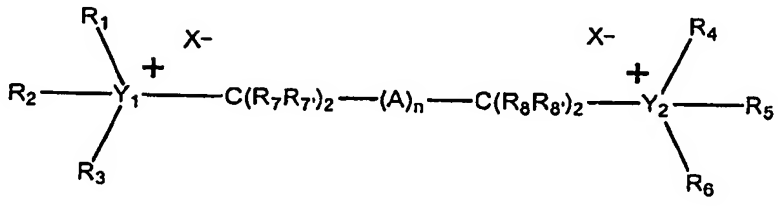
In another embodiment of the invention the compound of formula (I) is:



In a further embodiment of the invention the compound of formula (I) is:



Disclosed herein is a compound of formula (II), wherein said compound of formula (II) has the structure



(II)

wherein

Y_1 and Y_2 may be the same or different and are selected from N or P;

X- is a counterion;

R_1 to R_6 may be the same or different and are independently selected from the group consisting of optionally substituted C_{1-10} alkyl, optionally substituted C_{2-10} alkenyl, optionally substituted C_{2-10} alkynyl, optionally substituted C_{3-10} cycloalkyl, optionally substituted aryl and optionally substituted aralkyl, wherein said substituents are independently selected from C_{1-3} alkyl, hydroxyl and halogen; or

R_1 and R_2 together with the Y_1 group to which they are attached may optionally form an heterocycloalkyl group; and R_4 and R_5 together with the Y_2 group to which they are attached may optionally form a heterocycloalkyl group; wherein said heterocycloalkyl group may be optionally substituted with one or more groups selected from C_{1-3} alkyl, hydroxyl and halogen;

each A is independently selected from CR_9R_{9a}, optionally substituted phenyl, and optionally substituted C_{5-7} cycloalkyl, wherein said substituents are independently selected from C_{1-3} alkyl, hydroxyl and halogen;

n is an integer from 4 to 18 or 5 to 18 or optionally 7 to 16;

R_7, $R^{7'}$, R^8 and $R^{8'}$ may be the same or different and are independently selected from hydrogen, F and Cl;

R_9 and R_{9a} may be the same or different and are independently selected from the group consisting of hydrogen, halogen, COR_{10}, OR_{11}, SR_{11}, $NR_{12}R_{13}$, $CON_{12}R_{13}$, OH, SH, amino acids, dipeptidyl, tripeptidyl, tetrapeptidyl and pentapeptidyl;

R_{10} is OH or OR_{11};

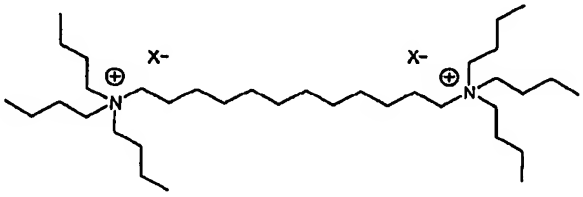
R_{11} is selected from the group consisting of optionally substituted C_{1-10} alkyl, optionally substituted C_{2-10} alkenyl, optionally substituted C_{2-10} alkynyl, optionally substituted C_{3-10} cycloalkyl, and optionally substituted aralkyl, wherein said substituents are independently selected from C_{1-3} alkyl, hydroxyl and halogen;

R_{12} and R_{13} are independently selected from the group consisting of hydrogen, optionally substituted C_{1-10} alkyl, optionally substituted C_{2-10} alkenyl, optionally substituted C_{2-10} alkynyl, optionally substituted C_{3-10} cycloalkyl, optionally substituted heterocycloalkyl, optionally substituted heteroaralkyl, and optionally substituted aralkyl, wherein said substituents are independently selected from C_{1-3} alkyl, hydroxyl, halogen, and $C(O)OR_{11}$; or R_{12} and R_{13} together with the N to which they are attached may optionally form a heterocycloalkyl group.

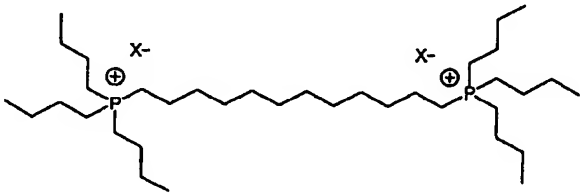
According to a second aspect of the invention there is provided the use of at least one compound of formula (II) for the manufacture of a medicament for the treatment, inhibition or prevention of a microbial infection

According to a third aspect of the invention there is provided a method of treating, inhibiting, or preventing a microbial infection in a vertebrate, said method comprising administering to said vertebrate an effective amount of at least one compound of formula (II).

In one embodiment of the invention the compound of formula (II) is:



In another embodiment of the invention the compound of formula (II) is:



According to a fourth aspect of the invention there is provided an antimicrobial composition comprising at least one compound of formula (I) according to the first aspect of the invention or formula (II), together with an industrially or pharmaceutically suitable carrier, diluent or carrier.

With reference to the fourth aspect of the invention, the composition may be one or more of antifungal, antibacterial, antiviral and antiparasitic. For example, in one embodiment the composition is an antifungal composition.

According to a fifth aspect of the invention there is provided a pharmaceutical composition comprising at least one compound of formula (I) according to the first aspect of the invention or formula (II) together with a pharmaceutically effective carrier, adjuvant or diluent.

According to a sixth aspect of the invention there is provided a method of inhibiting phospholipase in an organism comprising contacting said organism with an effective amount of at least one compound of formula (I) according to the first aspect of the invention or at least one compound according to formula (II), or a composition according to the third or fourth aspect of the invention.

With reference to the sixth aspect of the invention, the organism may be a microbial organism, such as bacteria, fungi, virus, and parasites, including for example protozoa. The phospholipase may be Phospholipase B.

According to a seventh aspect of the invention there is provided a method for identifying an antimicrobial agent comprising contacting microbial cells with a compound suspected of having antimicrobial properties, determining whether said compound inhibits a microbial phospholipase enzyme, wherein inhibition of said phospholipase enzyme indicates antimicrobial activity, and thereby identifying an antimicrobial agent.

In accordance with an eighth aspect of the invention there is provided a compound according to the first aspect of the invention or a compound according to the second aspect of the invention or a composition according to the fourth or fifth aspect of the invention when used as an antimicrobial agent.

With reference to any one of the second, third, seventh or eighth aspects of the invention, the microbe(s) may be bacteria, fungi, viruses or parasites, including protozoa.

In accordance with the present invention, the vertebrate may be human, non-human primate, murine, bovine, ovine, porcine, equine, caprine, leporine, avian, feline or canine.

Definitions

The following are some definitions that may be helpful in understanding the description of the present invention. These are intended as general definitions and should in no way limit the scope of the present invention to those terms alone, but are put forth for a better understanding of the following description.

In the context of this specification, the term "comprising" means "including principally, but not necessarily solely". Furthermore, variations of the word "comprising", such as "comprise" and "comprises", have correspondingly varied meanings.

In the context of this specification, the term "amino acid" is defined as having at least one primary, secondary, tertiary or quaternary amino group, and at least one acid group, wherein the acid group may be a carboxylic, sulfonic, or phosphonic acid, or mixtures thereof. The amino groups may be "alpha", "beta", "gamma" ... to "omega" with respect to the acid group(s). The backbone of the "amino acid" may be substituted with one or more groups selected from halogen, hydroxy, guanido, heterocyclic groups. Thus term "amino acids" also includes within its scope glycine, alanine, valine, leucine, isoleucine, methionine, proline, phenylalanine, tryptophane, serine, threonine, cysteine, tyrosine, asparagine, glutamine, asparte, glutamine, lysine, arginine and histidine, taurine,

betaine, N-methylalanine etc. (L) and (D) forms of amino acids are included in the scope of this invention.

The term "alkyl" as used herein, includes within its meaning monovalent or divalent, saturated, straight and branched chain hydrocarbon radicals.

The terms "alkenyl" and "alkenylene" as used herein, includes within its meaning, monovalent or divalent, straight and branched chain hydrocarbon radicals having at least one double bond.

The terms "alkynyl" and "alkynylene" as used herein, includes within its meaning, divalent, straight or branched chain hydrocarbon radicals having at least one triple bond.

The term "aryl" as used herein, includes within its meaning monovalent or divalent, single, polynuclear, conjugated and fused aromatic hydrocarbon radicals.

The term "aralkyl" as used herein, includes within its meaning monovalent or divalent, single, polynuclear, conjugated and fused aromatic hydrocarbon radicals attached to divalent, saturated, straight and branched chain hydrocarbon radicals.

The term "cycloalkyl" as used herein, includes within its meaning monovalent or divalent, saturated, monocyclic, bicyclic, polycyclic or fused polycyclic hydrocarbon radicals.

The term "cycloalkenyl" as used herein, includes within its meaning monovalent or divalent, saturated, monocyclic, bicyclic, polycyclic or fused polycyclic hydrocarbon radicals having at least one double bond.

The term "halo" or "halogen" as used herein, includes fluoro, chloro, bromo and iodo.

The term "heteroaryl" as used herein, includes within its meaning monovalent or divalent, single, polynuclear, conjugated and fused aromatic radicals having 1 to 12 atoms wherein 1 to 6 atoms are heteroatoms selected from O, N and S.

The term "heterocycloalkyl" as used herein, includes within its meaning monovalent or divalent, saturated, monocyclic, bicyclic, polycyclic or fused radicals wherein 1 to 5 atoms are heteroatoms selected from O, N or S.

The term "heterocycloalkenyl" as used herein, includes within its meaning monovalent or divalent, saturated, monocyclic, bicyclic, polycyclic or fused polycyclic radicals having at least 1 double bond and wherein 1 to 5 atoms are heteroatoms selected from O, N or S.

The term "therapeutically effective amount" as used herein, includes within its meaning a sufficient amount a compound or composition of the invention to provide the desired therapeutic or diagnostic effect. The exact amount required will vary from subject

to subject depending on factors such as the species being treated, the age and general condition of the subject, the severity of the condition being treated, the particular agent being administered and the mode of administration and so forth. Thus, it is not possible to specify an exact "effective amount". However, for any given case, an appropriate "effective amount" may be determined by one of ordinary skill in the art using only routine experimentation.

The term "substantially non-polar" as used herein, refers to groups which do not contain more than one polar substituent capable of forming hydrogen bonds and which do not undergo protonation/deprotonation at physiological pH.

The term "antimicrobial" includes within its scope antifungal, antibacterial, antiviral and antiparasitic.

The term "antifungal" as used herein and unless stated to the contrary, includes within its scope fungistatic activity and fungicidal activity.

The term "antibacterial" as used herein and unless stated to the contrary, includes within its scope bacteriostatic activity and bacteriocidal activity.

The term "antiviral" as used herein and unless stated to the contrary, includes within its scope virostatic activity and virocidal activity.

The term "Phospholipase B" as used herein refers to protein(s) having one or more activities selected from phospholipase B activity, lysophospholipase activity and lysophospholipase transacylase activity. The protein(s) may or may not have other enzyme activities. This term encompasses cell associated (intracellular and membrane bound) and secretory Phospholipase B enzyme.

Abbreviations

"PLB" – Phospholipase B activity

"LPL" – lysophospholipase activity

"LPTA" – lysophospholipase transacylase activity

"MIC" – minimum inhibitory concentration

"EDTA" – ethylenediaminetetraacetic acid

"EGTA" – ethyleneglycoldiamine tetraacetic acid

Brief Description of the Drawings

A preferred form of the present invention will now be described by way of example with reference to the accompanying drawings wherein:

Figure 1. Sites of action of the three activities of cryptoccocal Phosphopliase B: Phospholipase B

activity (PLB), Lysophospholipase activity (LPL), Lysophospholipase transacylase activity (LPTA)

Figure 2. Structures of example bis-cationic compounds.

Figure 3. Effects of protein concentration on membrane-associated phospholipase activities. Points shown are the means and SEM of three assays.

Figure 4. Effects of substrate concentration on cytosolic phospholipase activities. Points shown are means and SEM of three assays.

Figure 5. Effects of pH on membrane-associated phospholipase activities. In A, LPL/LPTA was measured using 30 sec incubation; in C, LPL/LPTA was measured using 10 min incubation. In B, PLB was measured using 10 min incubation. Points shown are means and SEM of three assays.

Figure 6. PLA and PLD activity versus pH.

Detailed Description of the Invention

The present invention relates to bis-ammonium and bis-phosphonium compounds and their use as antimicrobial agents, eg, antibacterial, antifungal, antiviral, antiparasitic and/or antiprotozoal agents. Compound(s) according to the invention may display broad spectrum activity. The bis-cationic compounds disclosed herein may be capable of inhibiting microbial phospholipase(s), however the present invention is not limited to bis-cationic compounds which are phospholipase inhibitors. Compounds which are capable of inhibiting intracellular phospholipases, eg, cytosolic or membrane associated phospholipases may have antimicrobial properties. Inhibition of extracellular phospholipases may have fungistatic properties and/or bacteriostatic, and/or virostatic.

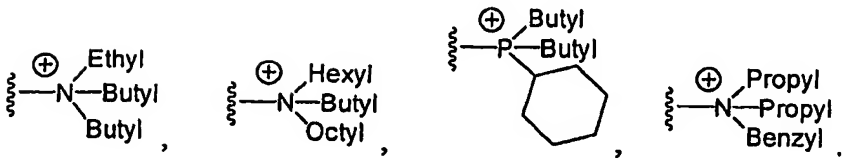
Compounds according to the invention generally comprise two cationic head groups substituted with non-polar or substantially non-polar groups, linked by a non-polar or substantially non-polar spacer group.

The cationic head groups may be the same or different and are independently selected from a quaternary ammonium group or a quaternary phosphonium group. Thus, the cationic head groups may both be quaternary ammonium groups, one may be a quaternary ammonium group and the other a phosphonium group, or both may be phosphonium groups.

The spacer may be from 7 to 20 atoms in length for Formula (I) compounds or from 6 to 20 atoms in length for Formula (II) compounds. For example, the spacer may be 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 atoms in length for formula (I) compounds or 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 atoms in length for formula (II) compounds. The spacer may comprise optionally substituted alkyl, alkenyl, alkynyl, cycloalkyl and aryl groups. Examples of optional substituents include one or more groups selected from halogen, carboxylic acid, esters, secondary or tertiary amines, secondary or tertiary amides, hydroxyl, thiol, amino acids, small peptides (eg, 2, 3, 4 or 5 amino acids in length), etc. According to the present invention, the carbon atoms at opposite ends of the spacer group which are immediately adjacent the respective cationic groups (sometimes referred to herein as the "α" and "ω" carbons) may independently be $-CH_2-$, $-CCl_2-$, -CFCl-, or $-CF_2-$ groups.

Compounds according to the invention may include one or more amino acids (as defined herein) or oligoamino acids (eg, 2, 3, 4, or 5 amino acids in length), attached to one or more carbons of the spacer portion of the compound. In the context of this specification, "oligoamino acids" refers to amino acids linked through their respective amino and acid groups to form dimers, trimers, tetramers, and pentamers. Compounds according to the invention may further optionally include one or more "depsi"(peptides), viz, peptides comprising an ester bond (eg, via a hydroxyl group in an amino acid or hydroxy acid such as glycollic acid, lactic acid, etc). α, β, or γ Amino acids may be used and (L) and (D) isomers may be used. Examples of amino acid substituents include glycinyl, alaninyl, valinyl, leucinyl, isoleucinyl, methioninyl, prolinyl, phenylalaninyl, tryptophanyl, serinyl, threoninyl, cysteinyl, tyrosinyl, asparaginyl, glutaminyl, aspartyl, glutamyl, lysinyl, argininyl and histidinyl. Other amino acids are known to those skilled in the art. The amino acid groups may be N- or C-linked to the spacer portion of the compound. The amino acid group may also be attached via the amino acid side chain, eg, a –COOH substituent of asparatic acid or glutamic acid, a –SH group of methionine or cysteine, etc.

The groups attached to the cationic quaternary N or P are non-polar or substantially non-polar and may be the same or different. Different groups attached to a respective cationic N or P may vary in length from each other, for example, by 2 to 6 carbons. For example, a cationic head group may comprise:



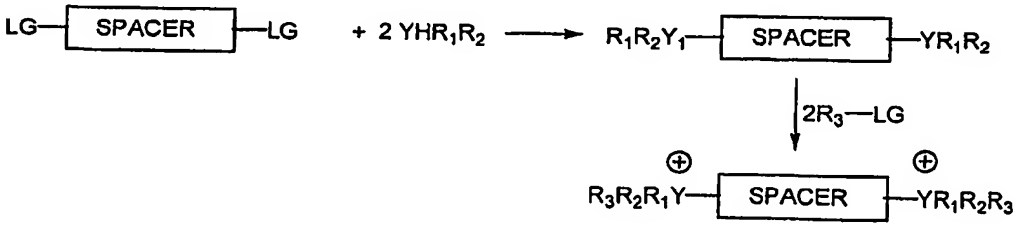
The "R_1 to R_6" groups attached to the cationic quaternary N and P atoms are generally non-polar or substantially non-polar. Thus, the "R_1 to R_6" groups generally do not contain more than one polar group which could be involved in hydrogen bonding. Thus, for example, respective "R_1 to R_6" groups may each be substituted with one hydoxyl group. In addition, generally, the "R_1 to R_6" groups do no contain groups which can become protonated to form positive charges or which can become disassociated to form negative charges in a pH range of about 4 to about 9.

Synthesis of Compounds

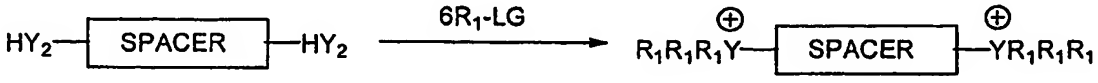
Compounds of formulae (I) and (II) may be prepared by methods known to those skilled in the art. Suitable methods are generally described, for example, and intermediates thereof are described, for example, in Houben-Weyl, *Methoden der Organischen Chemie;* J. March, *Advanced Organic Chemistry*, 4th Edition (John Wiley & Sons, New York, 1992); D. C. Liotta and M. Volmer, eds, *Organic Syntheses Reaction Guide* (John Wiley & Sons, Inc., New York, 1991); R. C. Larock, *Comprehensive Organic Transformations* (VCH, New York, 1989), H. O. House, *Modern Synthetic Reactions* 2nd Edition (W. A. Benjamin, Inc., Menlo Park, 1972).

The present invention includes within its scope all isomeric forms of the compounds disclosed herein, including all diastereomeric isomers, racemates and enantiomers. Thus, as appropriate, formulae (I) and (II) should be understood to include, for example, *cis*, *trans*, (R), (S), Δ, Λ, (L), (D), (+), and/or (-) forms of the compounds.

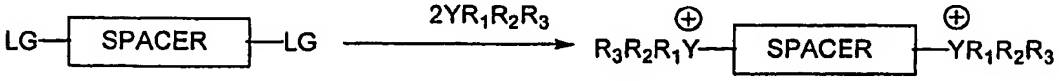
By way of example, compounds of formula (I) and (II) may be prepared according to the following general Schemes (a) – (f):



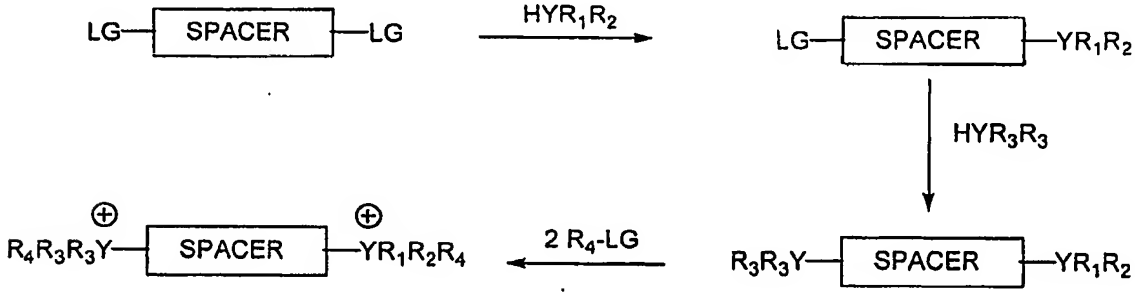
Scheme (a)



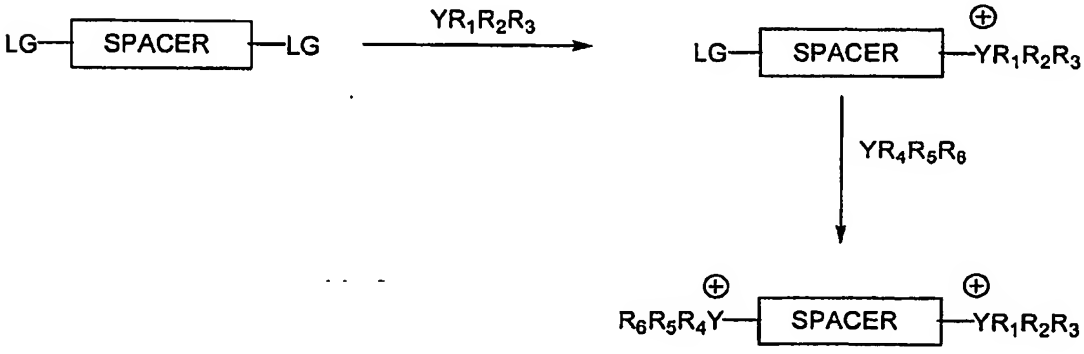
Scheme (b)



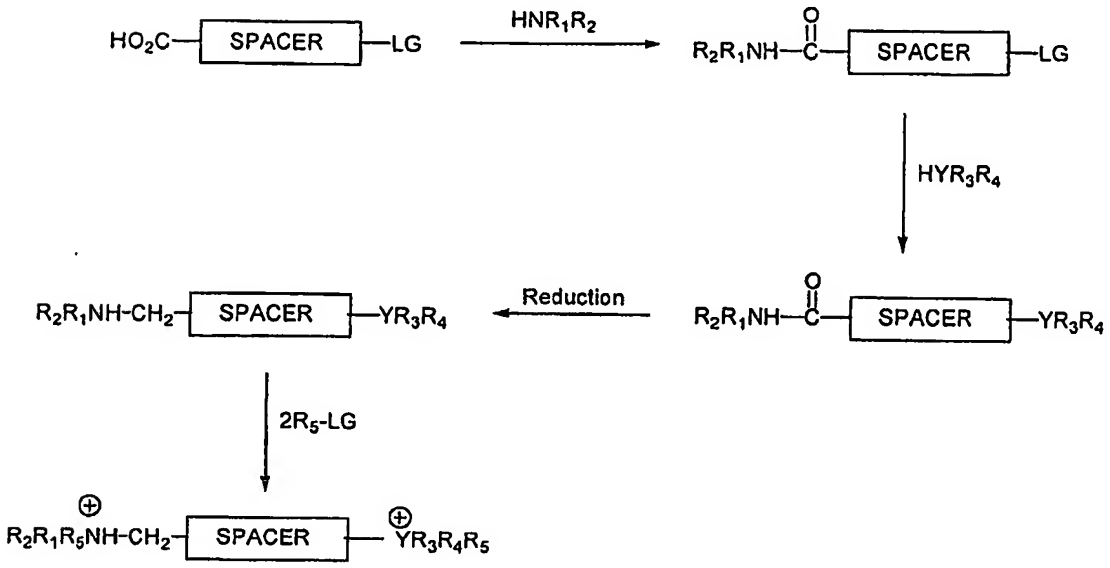
Scheme (c)



Scheme (d)



Scheme (e)



Scheme (f)

With reference to the above Schemes (a)-(f), Y represents N or P. LG represents a leaving group which can be displaced by a nucleophilic amine or phosphine. Examples of suitable leaving groups are known those skilled in the art and include, for example, chloro, bromo, iodo, mesylate, tosylate and triflate groups. The various leaving groups in the above Scheme may be the same or different and may be varied as appropriate to modify the reactivity. In the above Scheme, the groups R_1, R_2, R_3, R_4, R_5 and R_6 represent non-polar or substantially non-polar groups, for example, alkyl groups, cycloalkyl groups, alkenyl groups, aralkyl groups such as benzyl, etc. Respective R_1, R_2, R_3, R_4, R_5 and R_6 groups may be the same or different and each may be optionally substituted, e.g, with lower alkyl groups, hydroxyl and halogen substituents. Scheme (a) illustrates a synthetic route for a symmetrical bis cationic compound. However, those skilled in the art will realise that by varying the stoichiometric ratios and reaction conditions, or by varying the R_1. R_2. and R_3. groups. asymmetric compounds may also be prepared according to the above route.

With reference to Scheme (f), suitable reducing agents are known to those skilled in the art and include, for example, H_2, Pd/C, $LiALH_4$, etc.

With reference to Schemes (a) – (f) above, suitable reagents, reaction conditions etc, will be known to those skilled. By way of example, suitable solvents may include relatively polar solvents, such as acetonitrile, ethanol, ethers, methylisobutyl alcohol,

methylisobutyl ketone, and the like. The reaction temperature(s) may be adjusted as appropriate to control the rate of reaction. By way of example, suitable reaction temperatures are generally above room temperature, eg, greater than 50°C.

Pharmaceutical and/or Therapeutic Formulations

Compounds according to the present invention may be useful for the treatment, inhibition or prevention of microbial infection, such as fungal, bacterial, viral and protozoal infection. The compounds according to the present invention may also be useful as disinfectants.

Types and classes of microbes are known in the art. Classes of microbes are listed, for example, in *Manual of Clinical Microbiology*, 7th Edition, 1999, American Society of Microbiology, the entire contents of which are incorporated herein by reference. Some general examples of microorganisms include the following, however it is to be understood that the scope of the invention is by known means limited to these microorganisms:

Bacteria including: Gram positive cocci, such as *Staphylococcus spp.*, *Staphylococcus spp.*, *Streptococcus spp.*, *Enterococcus spp.*; gram positive rods, such as Coryneform,*Listeria spp.*, *Erysipelothrix spp.*,and *Kurthia spp.*, *Bacillus spp.*, *Mycobacterium spp.*; Gram negative bacteria, such as *Enterobacteriaceae sp.*, *Escherichia spp.*, *Shigella spp.*, *Salmonella spp.*, *Klebsiella spp.*, *Enterobacter spp.*, *Citrobacter spp.*, *Serratia spp.*, *Aeromonas spp.* and *Plesiomonas spp.*, *Pseudomonas spp.*; *Acinetobacter spp.*, *Alcaligenes spp.*, *Moraxella spp.* and *Methylobacterium spp*; *Actinobacillus spp.*, *Capnocytophaga spp.*, *Eikenella spp.*, *Kingella spp.*, such as *Legionella spp.*, *Neisseria spp.*, *Branhamella spp.*; anaerobic bacteria, including *Clostridium spp.*, *Peptostreptococcus spp.*, *Propionibacterium spp.*, *Lactobacillus spp.*, *Actinomyces spp.*; *Bacteroides spp.*,, *Porphyromonas spp.*, *Prevotella spp.*, *Fusobacterium spp.*, and other anaerobic gram negative cocci; curved and spiral-shaped gram negative rods, including *Helicobacter spp.*, *Borrelia spp.*; *Mycoplasmas* and obligate intracellular bacteria, such as *Mycoplasma spp.*, *Ureaplasma spp.*, Chlamydia spp., Coxiella spp.;

Viruses including Human Immunodeficiency viruses (HIV), Human T-Cell *Lymphotropic Virus Types I* and *II*, *Herpes Simplex Viruses*, Human *cytomegalovirus*, *Varicella-Zoster Virus*, Human *Herpesviruses 6*, *7* and *8* and *Herpes B virus*, *Measles Virus*, *Mumps virus*, *Adenoviruses*, *Rhinoviruses*, *Rotaviruses*, *Hepatitis B* and *D viruses*, *Hepatitius C* and *G viruses*, Human *papillomavirus*;

Fungi include *Candida spp.*, *Cryptococcus spp.* and medically important yeasts, *Pneumocystis carini spp.*, *Aspergillus spp.*, *Fusarium spp.* and other Moniliaceous fungi,

Rhizopus spp., Rhizomucor spp., Absidia spp. and other agents of systemic and subcutaneous Zygomycoses, *Trichophyton spp., Microsporum spp., Epidermophyton spp., Bipolaris spp., Exophiala spp., Scedosporium spp., Sporothrix spp.* and other dematiaceous fungi, fungi causing Eumycotic Mycetoma;

Parasites include *Plasmodium spp., Babesia spp., Leishmania spp., Trypanosoma spp., Toxoplasma giardia spp.*, pathogenic and opportunistic free-living Amebae, intestinal and urogenital Amebae, Flagellates and Ciliates; Cryptosporidium, Cyclospora, Isospora, Microsporidia, and intestinal Helminths.

In accordance with the present invention, when used for the treatment or prevention of microbial infection, compound(s) of the invention may be administered alone. Alternatively, the compounds may be administered as a pharmaceutical formulation which comprises at least one compound according to the invention. The compound(s) may also be present as suitable pharmaceutically acceptable salts.

In accordance with the present invention, the compounds of the invention may be used in combination with other known treatments or antimicrobial agents, including antifungal treatments, antibiotics, disinfectants, etc. Suitable agents are listed, for example, in the Merck Index, *An Encyclopoedia of Chemicals, Drugs and Biologicals*, 12th Ed.,1996, the entire contents of which are incorporated herein by reference.

Combinations of active agents, including compounds of the invention, may be synergistic.

By pharmaceutically acceptable salt it is meant those salts which, within the scope of sound medical judgement, are suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like, and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well known in the art.

For instance, suitable pharmaceutically acceptable salts of compounds according to the present invention may be prepared by mixing a pharmaceutically acceptable acid such as hydrochloric acid, sulfuric acid, methanesulfonic acid, succinic acid, fumaric acid, maleic acid, benzoic acid, phosphoric acid, acetic acid, oxalic acid, carbonic acid, tartaric acid, or citric acid with the compounds of the invention. Suitable pharmaceutically acceptable salts of the compounds of the present invention therefore include acid addition salts.

For example, S. M. Berge *et al.* describe pharmaceutically acceptable salts in detail in *J. Pharmaceutical Sciences,* 1977, *66*:1-19. The salts can be prepared *in situ* during the final isolation and purification of the compounds of the invention, or separately by

reacting the free base function with a suitable organic acid. Representative acid addition salts include acetate, adipate, alginate, ascorbate, asparate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, digluconate, cyclopentanepropionate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide. 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium potassium, calcium, magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, triethanolamine and the like.

Convenient modes of administration include injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, topical creams or gels or powders, or rectal administration. Depending on the route of administration, the formulation and/or compound may be coated with a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the therapeutic activity of the compound. The compound may also be administered parenterally or intraperitoneally.

Dispersions of the compounds according to the invention may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, pharmaceutical preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injection include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Ideally, the composition is stable under the conditions of manufacture and storage and may include a preservative to stabilise the composition against the contaminating action of microorganisms such as bacteria and fungi.

In one embodiment of the invention, the compound(s) of the invention may be administered orally, for example, with an inert diluent or an assimilable edible carrier. The compound(s) and other ingredients may also be enclosed in a hard or soft shell

gelatin capsule, compressed into tablets, or incorporated directly into an individual's diet. For oral therapeutic administration, the compound(s) may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Suitably, such compositions and preparations may contain at least 1% by weight of active compound. The percentage of the compound(s) of formula (I) and/or (II) in pharmaceutical compositions and preparations may, of course, be varied and, for example, may conveniently range from about 2% to about 90%, about 5% to about 80%, about 10% to about 75%, about 15% to about 65%; about 20% to about 60%, about 25% to about 50%, about 30% to about 45%, or about 35% to about 45%, of the weight of the dosage unit. The amount of compound in therapeutically useful compositions is such that a suitable dosage will be obtained.

The language "pharmaceutically acceptable carrier" is intended to include solvents, dispersion media, coatings, anti-bacterial and anti-fungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the compound, use thereof in the therapeutic compositions and methods of treatment and prophylaxis is contemplated. Supplementary active compounds may also be incorporated into the compositions according to the present invention. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. "Dosage unit form" as used herein refers to physically discrete units suited as unitary dosages for the individual to be treated; each unit containing a predetermined quantity of compound(s) is calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The compound(s) may be formulated for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in an acceptable dosage unit. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

In one embodiment, the carrier may be an orally administrable carrier.

Another form of a pharmaceutical composition is a dosage form formulated as enterically coated granules, tablets or capsules suitable for oral administration.

Also included in the scope of this invention are delayed release formulations.

Compounds of the invention may also be administered in the form of a "prodrug". A prodrug is an inactive form of a compound which is transformed *in vivo* to the active

form. Suitable prodrugs include esters, phosphonate esters etc, of the active form of the compound.

In one embodiment, the compound may be administered by injection. In the case of injectable solutions, the carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by including various anti-bacterial and/or anti-fungal agents. Suitable agents are well known to those skilled in the art and include, for example, parabens, chlorobutanol, phenol, benzyl alcohol, ascorbic acid, thimerosal, and the like. In many cases, it may be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the analogue in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the analogue into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above.

Tablets, troches, pills, capsules and the like can also contain the following: a binder such as gum gragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it can contain, in addition to materials of the above type, a liquid carrier. Various other materials can be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules can be coated with shellac, sugar or both. A syrup or elixir can contain the analogue, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the analogue can be incorporated into sustained-release preparations and formulations.

Preferably, the pharmaceutical composition may further include a suitable buffer to minimise acid hydrolysis. Suitable buffer agent agents are well known to those skilled in the art and include, but are not limited to, phosphates, citrates, carbonates and mixtures thereof.

Single or multiple administrations of the pharmaceutical compositions according to the invention may be carried out. One skilled in the art would be able, by routine experimentation, to determine effective, non-toxic dosage levels of the compound and/or composition of the invention and an administration pattern which would be suitable for treating the diseases and/or infections to which the compounds and compositions are applicable.

Further, it will be apparent to one of ordinary skill in the art that the optimal course of treatment, such as the number of doses of the compound or composition of the invention given per day for a defined number of days, can be ascertained using convention course of treatment determination tests.

Generally, an effective dosage per 24 hours may be in the range of about 0.0001 mg to about 1000 mg per kg body weight; suitably, about 0.001 mg to about 750 mg per kg body weight; about 0.01 mg to about 500 mg per kg body weight; about 0.1 mg to about 500 mg per kg body weight; about 0.1 mg to about 250 mg per kg body weight; or about 1.0 mg to about 250 mg per kg body weight. More suitably, an effective dosage per 24 hours may be in the range of about 1.0 mg to about 200 mg per kg body weight; about 1.0 mg to about 100 mg per kg body weight; about 1.0 mg to about 50 mg per kg body weight; about 1.0 mg to about 25 mg per kg body weight; about 5.0 mg to about 50 mg per kg body weight; about 5.0 mg to about 20 mg per kg body weight; or about 5.0 mg to about 15 mg per kg body weight.

Alternatively, an effective dosage may be up to about 500mg/m^2. For example, generally, an effective dosage is expected to be in the range of about 25 to about 500mg/m^2, about 25 to about 350mg/m^2, about 25 to about 300mg/m^2, about 25 to about 250mg/m^2, about 50 to about 250mg/m^2, and about 75 to about 150mg/m^2.

Examples

The invention will now be described in more detail, by way of illustration only, with respect to the following examples. The examples are intended to serve to illustrate this invention and should not be construed as limiting the generality of the disclosure of the description throughout this specification.

Example 1 – Synthesis of Compounds

Example 1(a)

1,10-Bis(tributylammonium)decane dibromide (2)

Tributylamine (1.4 mL, 5.9 mmol) was added to a solution of 1,10-dibromodecane (0.50 g, 1.7 mmol) in anhydrous acetonitrile (2 mL) and the resulting solution was heated at reflux under an atmosphere of N_2 for 96 h. The solution was cooled and the solvent was removed under reduced pressure, then the residue was triturated with anhydrous ethyl acetate (5 x 20 mL) at –15 °C. The resulting oil was dissolved in the minimum amount of EtOH, then anhydrous diethyl ether (5 volumes) was added and the stirred mixture cooled to –78 °C to give **(2)** as a colourless precipitate which was collected by filtration (0.35 g, 31 %). ^{1}H NMR (d_6-DMSO, 200 MHz) δ 3.26 (m, 16H), 1.66 (br s, 16H), 1.38-1.24 (br m, 24H), 1.01 (t, *J* 7.1 Hz, 18H); m/z (ESI) 591 ($[M\text{-}Br^-]^+$, 46), 589 ($[M\text{-}Br^-]^+$, 51), 255 ($[M\text{-}2Br^-]^{2+}$, 100).

Example (1b)

1,12-Bis(tributylammonium)dodecane dibromide (3)

Tributylamine (1.4 mL, 5.9 mmol) was added to a solution of 1,12-dibromododecane (0.50 g, 1.5 mmol) in anhydrous acetonitrile (2 mL) and the resulting solution was heated at reflux under an atmosphere of N_2 for 96 h. The solution was cooled and the solvent was removed under reduced pressure, then the residue was triturated with anhydrous ethyl acetate (5 x 20 mL) at –15 °C. The resulting oil was dissolved in the minimum amount of EtOH, then anhydrous diethyl ether (5 volumes) was added and the stirred mixture cooled to –78 °C to give **(3)** as a colourless precipitate which was collected by filtration (0.31 g, 29 %), m.p. 84-85 °C. ^{1}H NMR (CD_3OD, 200 MHz) δ 3.13 (m, 16H), 1.50 (br s, 16H), 1.31-1.20 (br m, 28H), 0.89 (t, *J* 7.1 Hz, 18H); m/z (ESI) 619 ($[M\text{-}Br^-]^+$, 100), 617 ($[M\text{-}Br^-]^+$, 68), 269 ($[M\text{-}2Br^-]^{2+}$, 90).

Example 2 – Characterisation of Enzyme Activities

Cryptococcus neoformans is the commonest cause of fungal meningitis, which is fatal if untreated. Pathogenic strains of cryptococci produce a number of so-called "virulence factors", one of which is a secreted phospholipase, termed phospholipase B (EC 3.1.1.5). This phospholipase is a single protein containing three separate activities, including (i) phospholipase B activity (PLB), which removes both acyl chains simultaneously from phospholipids, (ii) lysophopholipase (LPL), which removes the single acyl chain from lysophospholipids, and (iii) lysophospholipase transacylase

(LPTA), which adds an acyl chain to lysophospholipids to form phospholipids (see Fig. 1.).

Secreted phospholipase B is involved in the survival of cryptococci in macrophages, destruction of lung tissue and production of eicosanoids, which modulate phagocytic activity. This Phospholipase B is also related to virulence in other medically important fungi such as *Candida albicans* and *Aspergillus fumigatus*. Consequently, secreted Phospholipase B enzyme may be a potentially useful target for treatment of microbial infection, including, for example, antibacterial, antifungal, antiviral and antiparasitic treatments.

The cell-associated (membrane and cytosolic) phospholipase B activities in *C. neoformans,* were characterised.

Example 2(a) – Materials and Methods

Fungal isolates and media. A virulent clinical isolate of *C. neoformans* var. *grubii* (serotype A), H99, which produces high levels of secreted phospholipase B activity was used for cell-associated phospholipase characterisation and inhibition of phospholipase activities. Isolate H99 was kindly supplied by Dr. Gary Cox (Duke University Medical Center, Durham, NC, USA), and subcultured onto Sabouraud dextrose agar (SDA) at 30°C.

Preparation of supernatants containing secreted phospholipase activities. Isolate H99 was grown to confluence on SDA in 16 cm diameter Petri dishes for 72 h at 30°C in air. Cells scraped from 10-20 dishes were washed sequentially with isotonic saline and imidazole buffer (10 mM imidazole, 2 mM $CaCl_2$, 2 mM Mg Cl_2, 56 mM D-Glucose, made up in isotonic saline, pH 5.5), resuspended in a volume of this buffer of about 10% of the cell volume, and incubated for 24 h at 37°C. The cell-free supernatant was separated by centrifugation as previously described and stored at -70°C.

Cellular disruption to prepare membrane and cytosolic fractions. The cell pellet from the preparation of the supernatant, above, was also frozen at –70°C. After washing twice with imidazole buffer, it was disrupted in the presence of a protease inhibitor cocktail (P 8215 for fungal and yeast cells; 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 100mM, 1,10-phenanthroline, 500mM, pepstatin A, 2.2mM, E-64, 1.4 mM; Sigma) in a MiniBeadbeater-8 Cell Disrupter (MBB-8; Daintree Scientific, Tasmania, Australia) for three cycles of 1 min, alternating with a 1 min cooling period on ice. The homogenate was centrifuged at 14,000g for 15 min to obtain the membrane (pellet) and the cytosolic (supernatant) fractions. The cytosolic enzyme activities were

stable during storage at -70°C for up to 3 months, whereas the membrane associated activities were less stable (maximum 5 weeks).

Radiometric assay method for phospholipases. Enzyme activities were measured as described previously in a final volume of 125 µL at 37°C. For the determination of secreted PLB activity, carrier dipalmitoyl phosphatidylcholine (DPPC, final concentration 800 µM) and 1,2-di[1-^{14}C] palmitoyl phosphatidylcholine (20,000 dpm) were dried under nitrogen and suspended in 125 mM imidazole acetate buffer (assay buffer, pH 4.0) by sonication using a Branson 450 sonifier. The reaction time was 22 min, using 1 µg total protein and PLB activity was determined by the rate of decrease of the radiolabelled PC substrate, with appearance of the label in free fatty acid. Variations to these conditions for the cytosolic and membrane fraction assays are shown in Table 1. This assay also simultaneously allows for the determination of phospholipase A, C and D activities. These activities were measured by the appearance of radiolabel from PC in Lyso PC, diacylglycerol and phosphatidic acid, respectively.

Secreted LPL and LPTA activities were measured simultaneously in a reaction mixture containing 1-[^{14}C]palmitoyl lyso-PC (25,000 dpm) and carrier lyso-PC (final concentration 200 µM) in assay buffer. The reaction time was 15 s with 1 µg of total protein and LPL activity was measured by the rate of loss of 1-[^{14}C]palmitoyl lyso-PC with release of radiolabelled fatty acids. LPTA activity was estimated from the rate of formation of radiolabelled PC. Variations to these conditions for membrane and cytosolic fractions are presented in Table 1.

All reactions were terminated by adding 0.5 mL of chloroform: methanol (2:1 v/v). The reaction products were extracted by the method as disclosed in Bligh, E.C. and W.J. Dyer. 1959 *'A rapid method of total lipid extraction and purification.'* Can. J. Biochem. Physiol. 37:911-917. , separated by TLC and quantified as previously described. In the case of PLC activity, the TLC plates were developed in petroleum ether (BP 60-80°C): diethyl ether: acetic acid (90:15:1, v/v/v) instead of chloroform: methanol: water (65:25:4, v/v/v).

Table 1: Optimal conditions for H99 cell-associated and secreted phospholipases

	Cytosolic		Membrane		Secreted	
Activity	LPL/LPTA	PLB	LPL/LPTA	PLB	LPL/LPTA	PLB
Protein (µg)	1	4	80	120	1	1
Time	20 s	18 min	30 s	18 min	15 s	22 min

Substrate (μM)	200	1000	600	800	200	400
PH	4.0	4.0	4.0	4.0	4.0	4.0

Example 2(b) - Characterisation of enzyme activities.

All experiments were carried out in duplicate or triplicate. The effects of pH on the various phospholipase activities were measured using 50 mM final concentration of imidazole-acetate buffer (pH range 3-5), MES buffer (pH range 6-8) and glycine buffer (pH 9-10). Controls for non-enzymic breakdown of substrates were included at all pH values. Cations, Triton X-100 and metal chelators were made up as stock solutions in water and diluted to the final concentration in the appropriate assay buffer.

Protein assays.

Total protein estimations were performed using a Coomassie Blue binding assay (for supernatant containing secreted enzymes) or the bicinchoninic acid (BCA kit) for cell-associated fractions, with BSA as standard (Pierce Chemical Co., IL, USA).

Identification of PLB, LPL and LPTA in cell-associated fractions.

Assays of cell-associated phospholipase activities were performed at pH 4.0, using the substrates palmitoyl lyso-phosphatidylcholine (Lyso-PC) and DPPC, since these compounds were preferred substrates of the secreted enzyme in both its natural and purified states and enzyme activity was maximal at pH 4.0. Hydrolysis of the substrate lyso-PC by the cryptococcal membrane and cytosolic fractions, resulted in the formation of free fatty acids and PC only, indicating the presence of both LPL and LPTA, as found for the secreted enzyme. Similarly, DPPC, radiolabelled in both acyl chains, was degraded to produce free fatty acids, only, indicating that the activity was due to PLB at pH 4.0.

Effect of protein concentration and time on phospholipase activity.

Activity of the cytosolic fraction with increasing protein concentration was linear only to 1 μg for LPL/LPTA and 4 μg for PLB, similar to values for the secreted enzyme (not shown). In contrast, membrane-associated LPL and LPTA activities were linear with increasing protein concentration to 80 μg, after which no further increase occurred (Fig. 3A). The PLB activity was linear to 160 μg protein (Fig. 3B).

The time course of both the cytosolic and membrane activities resembled that of the secreted enzyme, with linearity of LPL/LPTA only to 20-30 sec, beyond which no further increase occurred. Membrane-associated PLB activity was linear to 30 min, whereas cytosolic activity was linear to 22 min (not shown).

Effect of substrate concentration on enzyme activity.

Cytosolic LPL/LPTA activities reached a maximum between 50-200 μM Lyso-PC, after which there was a rapid decline in activity with increasing substrate concentration (Fig. 4A). Cytosolic PLB activity reached saturation at 400 μM DPPC, and declined after 1000 μM (Fig. 4B). Membrane-associated LPL/LPTA reached a maximum around 50 μM Lyso-PC, but maintained the same level of activity until 600 μM, after which it decreased (not shown). The membrane-associated PLB activity reached a maximum at 200 μM DPPC, and declined after 800 μM (not shown).

Effects of pH on enzyme activity.

Optimal conditions for further studies were selected from Table 1. Variations in cell-associated phospholipase activity were observed over the pH range 3-10. Cytosolic LPL/LPTA activities exhibited two pH optima, at 4.0 and 6.0, with activity decreasing to zero at pH 9.0 (not shown). The cytosolic phospholipase B pH profile (not shown) was similar to that of membrane-associated PLB (Fig. 5B), though less sharp. The pH optimum was 3-4 for both membrane-associated LPL and LPTA, and decreased gradually to zero at pH 9.0 (Fig 5A). The optimum pH for membrane PLB (pH 4.0) was very narrow (Fig. 5B), with virtually no activity at pH 7.0.

The most obvious difference between the cell associated (cytosolic and membrane-associated) activities and those of both the crude and purified secreted enzymes was the greater activity of the cell-associated enzymes at pH 6.0 (PLB) and 7-8 (LPL/LPTA). Interestingly, when the incubation time for the LPL/LPTA assay was extended from 20-30 sec to 10 min, the activity was increased over the range pH 7-9, but not at lower pH values, in both membrane and cytosolic enzyme preparations (Fig. 5 C, membrane-associated enzyme).

Cellular distribution of phospholipase B activities.

Taking 4.0 as the optimal pH for all three activities (Table 1), it is clear that the distribution of PLB differs from that of LPL and LPTA, in that the greatest percentage of the total activity is secreted (Table 2). With LPL and LPTA most of the activity is cytosolic. The specific activities and percentages of all three activities were lowest in the membrane fraction (Table 2).

Table 2. Cellular distribution of phospholipase activities measured at pH 4.0.

	[a] Specific Activity			[b] Total Activity			% Distribution		
	LPL	**LPTA**	**PLB**	**LPL**	**LPTA**	**PLB**	**LPL**	**LPTA**	**PLB**
Secreted	*86.6*	*53.9*	*2.1*	*650*	*405*	*16.2*#*	*36.1*	*29.9*	*82.7*#*
	(7.2)	*(3.5)*	*(0.29)*	*(54)*	*(26)*	*(2.1)*	*(1.4)*	*(1.9)*	*(10.7)*
Cytosolic	*78.4*	*68.9*	*0.2*	*955**	*839**	*2.5*	*53.1**	*62.0**	*12.8*
	(12.6)	*(20.6)*	*(0.15)*	*(154)*	*(251)*	*(1.8)*	*(8.6)*	*(18.6)*	*(9.2)*
Membrane	*0.54*	*0.31*	*0.003*	*195*	*109*	*0.9*	*10.8*	*8.1*	*4.6*
	(0.28)	*(0.15)*	*(0.001*	*(99)*	*(53)*	*(0.4)*	*(5.5)*	*(3.9)*	*(2.0)*

*Data are expressed as the means and SEM of at least three assays, with activities calculated as μmol substrate degraded or product formed (LPTA) per min per mg protein[a] or per total protein[b] in the cellular fraction. *P < 0.01, compared with membrane activity; #P < 0.01, compared with cytosolic activity (ANOVA).*

Modifying agents and cell-associated activities.

There was no stimulation of any of the cytosolic or membrane-associated activities by 10 mM calcium or magnesium at pH 4.0 (Table 3A). Triton X-100 was the only notable inhibitor at pH 4.0, with cytosolic and membrane-associated LPTA and PLB most affected (Table 3A).

When assayed at pH 7.0, all three of both cytosolic and membrane-associated activities were stimulated by calcium (Table 3B). Cytosolic PLB, and all three of the membrane-associated activities were also stimulated by magnesium (Table 3B). This was reflected in lower activity in the presence of EDTA and EGTA for cytosolic LPL and LPTA. Both $FeCl_3$ and Triton X-100 were inhibitors of all three activities, from both cytosolic and membrane-bound fractions. Triton X-100 was less effective in inhibiting the PLB, than the LPL and LPTA activities (Table 3B).

Table 3. Effects of modifying agents on cytosolic and membrane-associated activities.

(A) Assayed at pH 4.0[a]

Modifier	*Cytosolic*			*Membrane-associated*		
	LPL[b]	*LPTA*[b]	*PLB*	*LPL*[b]	*LPTA*[b]	*PLB*[b]
Calcium	*100*	*100*	*100*	*100*	*96*	*83*
Magnesium	*73*	*70*	*100*	*97*	*91*	*79*
EDTA	*100*	*100*	*100*	*98*	*94*	*87*
EGTA	*86*	*88*	*100*	*100*	*90*	*77*
$FeCl_3$	*81*	*78*	*79*	*97*	*75*	*92*
Triton X-100	*62*	*39*	*54**	*71*	*52*	*19*

(B) Assayed at pH 7.0[a]

	LPL[b]	*LPTA*[b]	*PLB*	*LPL*	*LPTA*	*PLB*
Calcium	*236*	*206*	*591**	*166**	*140**	*533**
Magnesium	*96*	*85*	*477**	*145**	*123**	*467**
EDTA	*30*	*32*	*100*	*96*	*100*	*67*
EGTA	*26*	*29*	*163*	*100*	*100*	*200*
$FeCl_3$	*22*	*21*	*61*	*61**	*45**	*2**
Triton X-100	*0.9*	*0*	*59*	*4**	*2**	*33**

*[a]Activities are expressed as percentages of the control, taken as 100%. Values are the means of triplicate or [b]duplicate assays. Final concentrations of modifying agents were 10mM, except for Triton X-100, which was 0.1%(w/v). *Significantly different from the controls, P <0.01, by the Dunnett Multiple Comparisons Test.*

Phospholipases A, C, and D.

Secreted forms of Phospholipases A, C, and D have not been identified in cryptococcal supernatants. High levels of phospholipase A (PLA) were detected by the formation of radiolabelled Lyso-PC from DPPC at pH 7-8 in membrane preparations (Fig. 6A), but only trace amounts of PLA were found in the cytosolic fraction (not shown). Small amounts of phospholipase D (PLD) activity were detected by the formation of radiolabelled phosphatidic acid from both cytosolic and membrane fractions at pH 7-8 (Fig. 6B, membrane PLD). No evidence of PLC was found at any pH value using DPPC as substrate.

Under the assay conditions tested, phospholipase B was the major phospholipase present in the cytosol and secreted from *C. neoformans*. In contrast, phospholipases A and D were membrane-associated, with some PLD activity in the cytosol. The pH optimum for PLB activity, whether secreted, membrane-bound or cytosolic, was always acidic (pH 4.0), whereas the cytosolic LPL/LPTA was bimodal (pH 4.0 and 6.0). PLA and PLD activities were detected only at pH 7-8. These observations are consistent with the role of secreted PLB activities in cryptococcal virulence since the putative sites of action of Phospholipase B are the acidic vacuoles of macrophage-like cell lines, and mouse macrophages *in vivo*.

The membrane-associated LPL/LPTA activities were stimulated by both calcium and magnesium (Table 3). The unexpected stimulation of cell-associated PLB activity by calcium and magnesium at pH 7.0 might be due in part to PLA activity.

Example 3
Selection and Testing of Potential Phospholipase Inhibitors

Example 3(a) – MATERIALS AND METHODS

Selection of potential phospholipase inhibitors. Selection of potential inhibitors was based on the traditional approach of testing compounds that are structurally related to the substrate, i.e. phospholipids. Commercially available compounds were initially used containing the two dominant features in phospholipids (one or two hydrophobic alkyl chains and a tetra-alkylated strongly positively charged nitrogen atom), which would be metabolically stable and sufficiently water-soluble to avoid use of solvents in the assays.

Preparation of inhibitors and use in assays. The following compounds were tested (for structures, see Fig. 2): compound 1, 1,12-bis(tributylphosphonium)dodecane dibromide (Fluka AG, Buchs, Switzerland); compound 2, 1,10-bis(tributylammonium)-decane dibromide; compound 3, 1,12-bis(tributylammonium)do-decane dibromide (both synthesised in-house); compound 4, 1,10-bis(trimethylammonium)decane dibromide ["decamethonium"] (Sigma, St.Louis MO, USA). All compounds were prepared as stock solutions of 700 μM in assay buffer containing 5 mM EDTA, which was then diluted serially with buffer to obtain solutions of 70, 7, 0.7 and 0.07 μM. In each assay, 45 μL of these solutions was used, and the final volume of 125 μL was made up of substrate, enzyme and buffer. The radiometric assay was carried out as above. Inhibition was calculated as the percent of substrates (DPPC or Lyso-PC) remaining in the case of PLB and LPL activities, or of DPPC produced, in the case of the LPTA activity. The amounts converted, or produced, in the inhibitor-free control were normalised to 100%, and the inhibition calculated against it. All assays were done in triplicate.

Pancreatic phospholipase assay. Porcine pancreatic phospholipase A_2 suspension in 3.2M ammonium sulfate (2.9 mg protein/mL, Sigma St.Louis MO, USA) was used. One part of well mixed enzyme suspension was added to 4 parts of buffer (10 mM Tris/HCl, pH 8.2; 10 mM $CaCl_2$. Activity and inhibition by test compounds was then measured by the radiometric method described in Example 2 for fungal PLB activity. However, 25 μL enzyme solution was used, and the reaction time was 1 hour. These conditions result in ~ 60% substrate conversion in the inhibitor-free control.

Antifungal susceptibility testing. The antifungal activity of the compounds was measured by a standard microdilution method. The minimal inhibitory concentration of each compound (MIC) was defined as that which produced no visible growth after 48 h of culture *(Candida)* and 72 h *(Cryptococcus)* at 35°C. The fungal strains tested included

Cryptococcus neoformans H99, Cryptococcus neoformans ATCC 90112 and *Candida albicans* ATCC 10231. All tests were performed in duplicate.

Ammonium- and phosphonium compounds were examined. The structures and common names of four compounds studied are shown in Figure 2. These compounds each have a strong positive charge and fatty acid-like hydrophobicity.

Assays were performed at pH 4 in the absence of added cations, using the optimised conditions summarised in Table 1. Under these conditions only PLB, LPL and LPTA activities (both secreted and cell-associated) were measured. Initially, compounds deemed to be potential inhibitors were assayed at 25 and 250 μM. Those showing some inhibition were then also assayed at 2.5 and 0.25 μM.

This bis-tributylphosphonium compound (1) inhibits PLB activity more so than LPL or LPTA. Importantly it inhibits the cytosolic as well as the secretory enzyme, while it has no effect on the membrane-bound enzyme (Table 4). Interestingly, it inhibits the porcine pancreatic PLA2 more strongly than the fungal secretory Phospholipase B (Table 5). This is in contrast to the two bis-tributylammonium compounds (2 and 3) which inhibit the fungal enzyme more strongly, and thus form a platform to achieve even higher selectivity. The fungicidal activity of these three tributyl bis-cationic compounds is quite strong, the best having an MIC of 2.5 μMolar (Table 6). There is a sharp drop in MIC as the chain length is increased by two CH_2 groups, and, importantly this increase in chain length also increases the inhibition potency (Table 5). Compound (4), which has only methyl-alkylation at the quaternary nitrogen, shows neither enzyme inhibition nor antifungal activity (Tables 4 and 5).

Table 4. Inhibition of the activities of C. neoformans strain H99 phospholipases by 1,12-bis(tributylphosphonium)dodecane dibromide (1)

Compound[a]	% Inhibition[b]											
	LPL				LPTA				PLB			
	0.25	2.5	25	250[c]	0.25	2.5	25	250[c]	0.25	2.5	25	250[c]
Secretory Enzyme												
1[a]	0	0	0	6.3* (0.8)	0	0	0	3.7* (0.6)	0	0	35.2* (1.5)	55.0* (1.5)
Cytosolic Enzyme												
1	n.d.	0	0	12.7* (0.9)	n.d.	0	0	0	n.d.	0	0	41.3* (2.3)

Membrane-bound Enzyme

1	n.d.	0	0	0	n.d.	0	0	0	n.d.	0	0	0

[a]The structures and abbreviations for these compounds are shown in Fig. 2. Compound 4 did not inhibit any of the activities of any enzyme at these concentrations.
[b]Data are expressed as the means and SEM (in brackets) of at least three assays.
**Significantly different from the inhibitor-free controls, P<0.01 by the Dunnett Multiple Comparison Test.*
[c]Concentrations are μM
n.d. = not determined.

Table 5: Inhibition[a)] of Secretory Cryptococcal H99 Phospholipase and ppPLA$_2$ by Dimeric Cationic Compounds.

Conc. (μM)	Test Compounds[b)]							
	1,10-bis(tributyl)ammonium decane (2)		1,12-bis(tributyl)ammonium dodecane (3)		1,12-bis(tributylphosphonium) dodecane (1)		1,10-bis(trimethylammonium) decane [decamethonium] (4)	
	PLB	ppPLA$_2$	PLB	ppPLA$_2$	PLB	ppPLA$_2$	PLB	ppPLA$_2$
250	90	65	85	86	55	97	0	0
25	80	25	80	36	35	62	0	0
2.5	30	0	65	0	0	5	0	0
0.25	0	0	20	0	0	0	n.d	n.d.

[a]Data are expressed as percentage inhibition of activity and are the means of at least three assays.
[b]Structures and abbreviations are given in Figure 2.
n.d. = not determined.

Example 4 - Antifungal Activity

Compounds **1-4** referred to in Example 3 were assayed for antifungal activity in a standardised serial dilution sensitivity test against two strains of *C. neoformans* and one strain of *Candida albicans* (Table 6). The two stronger phospholipase inhibitors (**1** and **3**) were quite potent, with MIC in the 2.5 to 10 μM range, whereas the non-inhibitory decamethonium compound (**4**) had a much higher MIC (88μM - 350 μM) (Table 6).

Table 6: MIC of synthesised Compounds Tested against C.neoformans reference strain ATCC 90112 and Candida albicans reference strain ATCC 10231

Name	FW	C. neof. ATCC 90112	C. albicans ATCC 10231
		µM	µM
Amphotericin B (control)	924	0.55	1.1
1,10-bis(Trimethylammonium)decane, dichloride ["Decamethonium"] (4)	418	88	>350
1,10-bis(Tributylammonium)decane, dibromide (2)	656	11	88
1,12-bis(Tributylammonium)dodecane, dibromide (3)	698	2.5	11
1,12-bis(Tributylphosphonium)dodecane, dibromide (1)	733	2.5	8.8

Example 5 – Pharmaceutical Formulations

The compounds of the present invention may be administered alone, although they may also be administered as a pharmaceutical formulation. For instance, the active ingredient may comprise, for topical administration, from 0.001% to 10% by weight, and more typically from 1% to 5% by weight of the formulation, although it may comprise as much as 10% by weight.

By way of illustration, specific examples of pharmaceutical compositions in accordance with the present invention are outlined below. The following are to be construed as merely illustrative examples of formulations and not as a limitation of the scope of the present invention in any way.

Example 5(a) - Topical Cream Composition

A typical composition for delivery as a topical cream is outlined below:

1,12-bis(Tributylphosphonium)dodecane, dibromide 1.0 g
Polawax GP 200 25.0 g
Lanolin Anhydrous 3.0 g
White Beeswax 4.5 g
Methyl hydroxybenzoate 0.1 g
Deionised & sterilised Water to 100.0 g

The polawax, beeswax and lanolin are heated together at 60°C, a solution of methyl hydroxybenzoate is added and homogenisation achieved using high speed stirring. The temperature is then allowed to fall to 50°C. The compound of the present invention, in this example being 1,12-bis(Tributylphosphonium)dodecane, dibromide, is then added and dispersed throughout, and the composition is allowed to cool with slow speed stirring.

Example 5(b) - Topical Lotion Composition

A typical composition for delivery as a topical lotion is outlined below:

1,12-bis(Tributylphosphonium)dodecane, dibromide 1.2 g
Sorbitan Monolaurate 0.8 g
Polysorbate 20 0.7 g
Cetostearyl Alcohol 1.5 g
Glycerin 7.0 g
Methyl Hydroxybenzoate 0.4 g

Sterilised Water about to 100.00 ml

The methyl hydroxybenzoate and glycerin are dissolved in 70 ml of the water at 75°C. The sorbitan monolaurate, polysorbate 20 and cetostearyl alcohol are melted together at 75°C and added to the aqueous solution. The resulting emulsion is homogenised, allowed to cool with continuous stirring and the 1,12-bis(tributylphosphonium)dodecane, dibromide is added as a suspension in the remaining water. The whole suspension is stirred until homogenised.

Example 5(c) - Eye Drop Composition

A typical composition for delivery as an eye drop is outlined below:

1,12-bis(Tributylphosphonium)dodecane, dibromide 0.3 g

Methyl Hydroxybenzoate 0.005 g

Propyl Hydroxybenzoate 0.06 g

Purified Water about to 100.00 ml.

The methyl and propyl hydroxybenzoates are dissolved in 70 ml purified water at 75°C, and the resulting solution is allowed to cool. 1,12-bis(tributylphosphonium)-dodecane, dibromide is then added, and the solution sterilised by filtration through a membrane filter (0.22 μm pore size), and aseptically packed into sterile containers.

Example 5(d) – Aerosol Composition

For an aerosol container with a capacity of 20-30 ml: a mixture of 10 mg 1,12-bis(tributylphosphonium)dodecane, dibromide with 0.5-0.8% by weight of a lubricating agent, such as polysorbate 85 or oleic acid, is dispersed in a propellant, such as freon, and put into an appropriate aerosol container for either intranasal or oral inhalation administration or topical application.

Example 5(e) - Composition for Parenteral Administration

A pharmaceutical composition of the present invention for intramuscular injection could be prepared to contain 1 mL sterile buffered water, and 1 mg of 1,12-bis(tributylammonium)dodecane, dibromide. Similarly, a pharmaceutical composition for intravenous infusion may comprise 250 ml of sterile Ringer's solution, and 5 mg 1,12-bis(tributylammonium)dodecane, dibromide.

Example 5(f) - Capsule Composition

A pharmaceutical composition of 1,12-bis(tributylammonium)dodecane, dibromide in the form of a capsule may be prepared by filling a standard two-piece hard gelatin

capsule with 50 mg of 1,12-bis(tributylammonium)dodecane, dibromide, in powdered form, 100 mg of lactose, 35 mg of talc and 10 mg of magnesium stearate.

Example 5(g) - Injectable Parenteral Composition

A pharmaceutical composition of this invention in a form suitable for administration by injection may be prepared by mixing 1% by weight of 1,12-bis(tributylammonium)dodecane, dibromide in 10% by volume propylene glycol and water. The solution is sterilised by filtration.

Example 5(h) - Ointment Composition

A typical composition for delivery as an ointment includes 1.0g of 1,12-bis(tributylphosphonium)dodecane, dibromide, together with white soft paraffin to 100.0 g, dispersed to produce a smooth, homogeneous product.

Example 5(i) - Gel Composition

A percutaneous gel can be prepared as outlined below:

1,12-bis(Tributylphosphonium)dodecane, dibromide 1.0 g

Propylene glycol 5.0 g

Isopropyl alcohol 20.0 g

Carboxyvinyl polymer 2.0 g

Aqueous ammonia q.s.

Purified water 72.0 g

The 1,12-bis(Tributylphosphonium)dodecane, dibromide is dissolved into a mixed solvent of propylene glycol and isopropyl alcohol. The Carboxyvinyl polymer is added to the solution after swelling in purified water. After stirring, the mixture is adjusted to pH 7 with the aqueous ammonia.

Example 5(j) – Powder Composition

A powder composition can be prepared by mixing 6.0 g of sodium caseinate, 3.0 g of xanthan gum and 60.0 g of water for 30 minutes at room temperature. To this aqueous phase 2.0 g of 1,12-bis(Tributylphosphonium)dodecane, dibromide is added, while agitating the solution to form an emulsion, having a pH around 7. The emulsion is homogenized and dehydrated by heating the emulsion to form dry powder particles. The dried powder is then sieved to obtain fine powder particles of mesh size 120-180μm.

Dated 13 November, 2003
The University of Sydney
Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

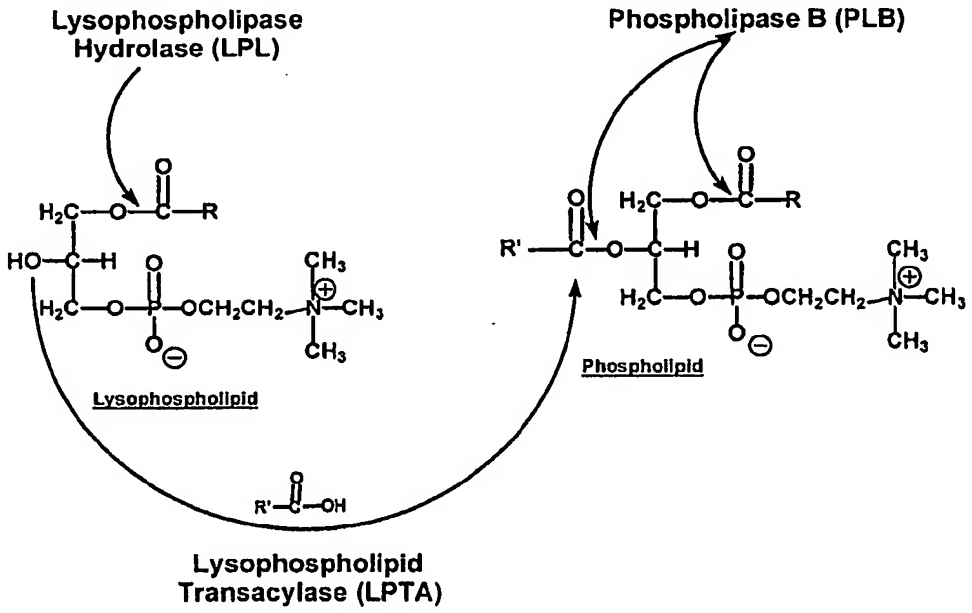
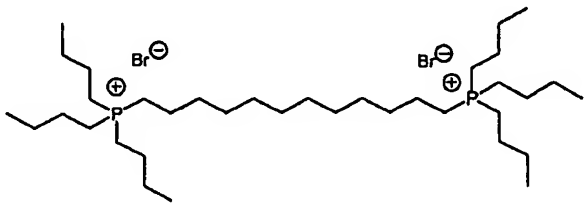
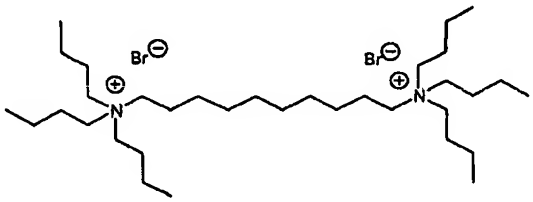

Lysophospholipase
Hydrolase (LPL)
Phospholipase B (PLB)
Lysophospholipid
Phospholipid
Lysophospholipid
Transacylase (LPTA)

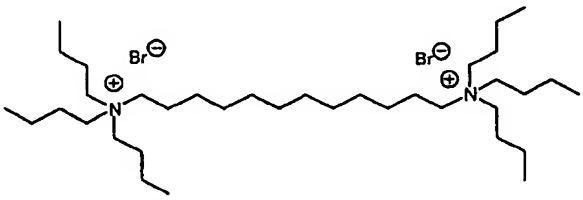
Figure 1



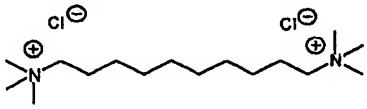
1,12-Bis(tributylphosphonium)dodecane dibromide **(1)**



1,10-Bis(tributylammonium)decane dibromide **(2)**



1,12-Bis(tributylammonium)dodecane dibromide **(3)**



1,12-Bis(trimethylammonium)decane dichloride("decamethonium") **(4)**

Figure 2

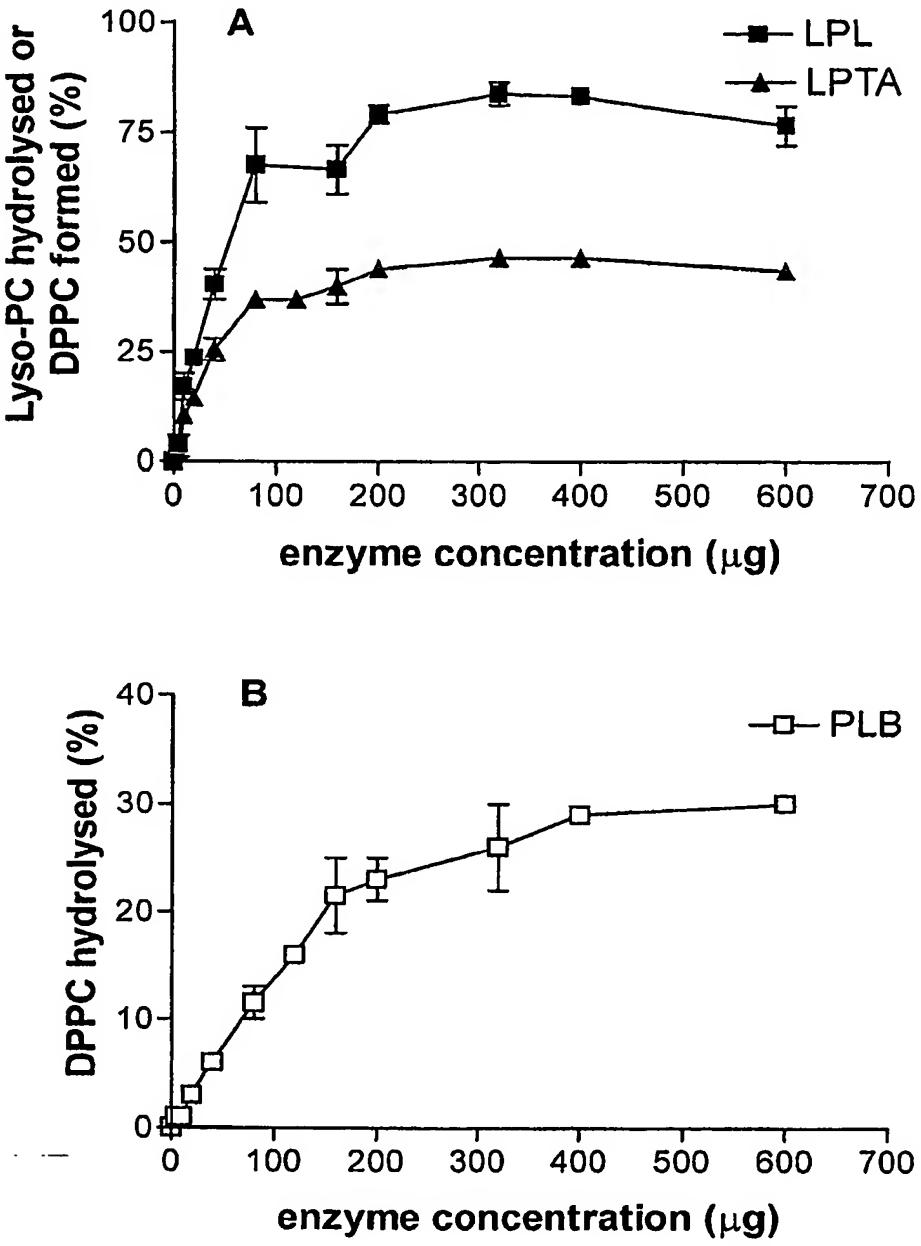

A
LPL
LPTA
Lyso-PC hydrolysed or DPPC formed (%)
100
75
50
25
0
0
100
200
300
400
500
600
700
enzyme concentration (μg)
B
PLB
DPPC hydrolysed (%)
40
30
20
10
0
0
100
200
300
400
500
600
700
enzyme concentration (μg)

Figure 3

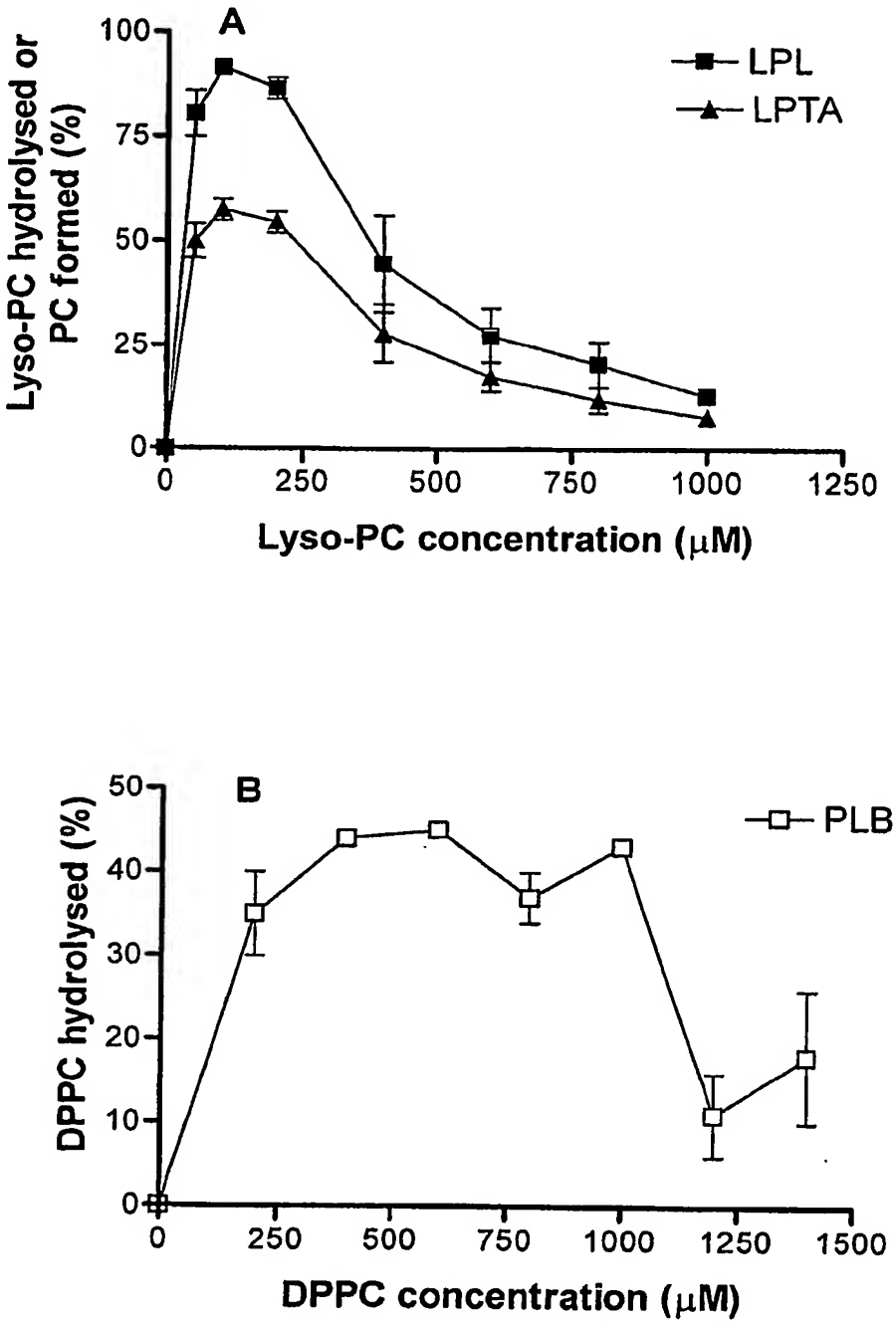

A
100
75
50
25
0
Lyso-PC hydrolysed or PC formed (%)
LPL
LPTA
0
250
500
750
1000
1250
Lyso-PC concentration (μM)
B
50
40
30
20
10
0
DPPC hydrolysed (%)
PLB
0
250
500
750
1000
1250
1500
DPPC concentration (μM)

Figure 4

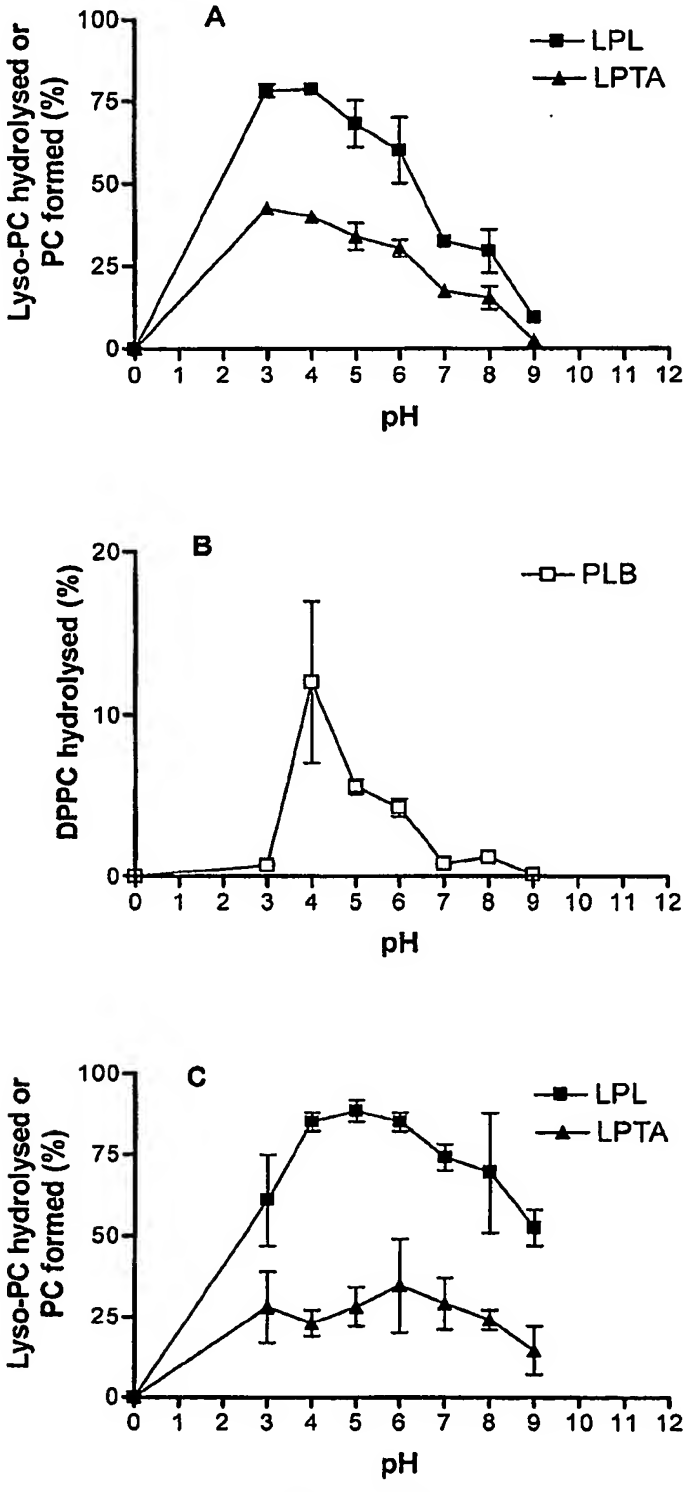

A
LPL
LPTA
Lyso-PC hydrolysed or PC formed (%)
100
75
50
25
0
0 1 2 3 4 5 6 7 8 9 10 11 12
pH
B
PLB
DPPC hydrolysed (%)
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
pH
C
LPL
LPTA
Lyso-PC hydrolysed or PC formed (%)
100
75
50
25
0
0 1 2 3 4 5 6 7 8 9 10 11 12
pH

Figure 5

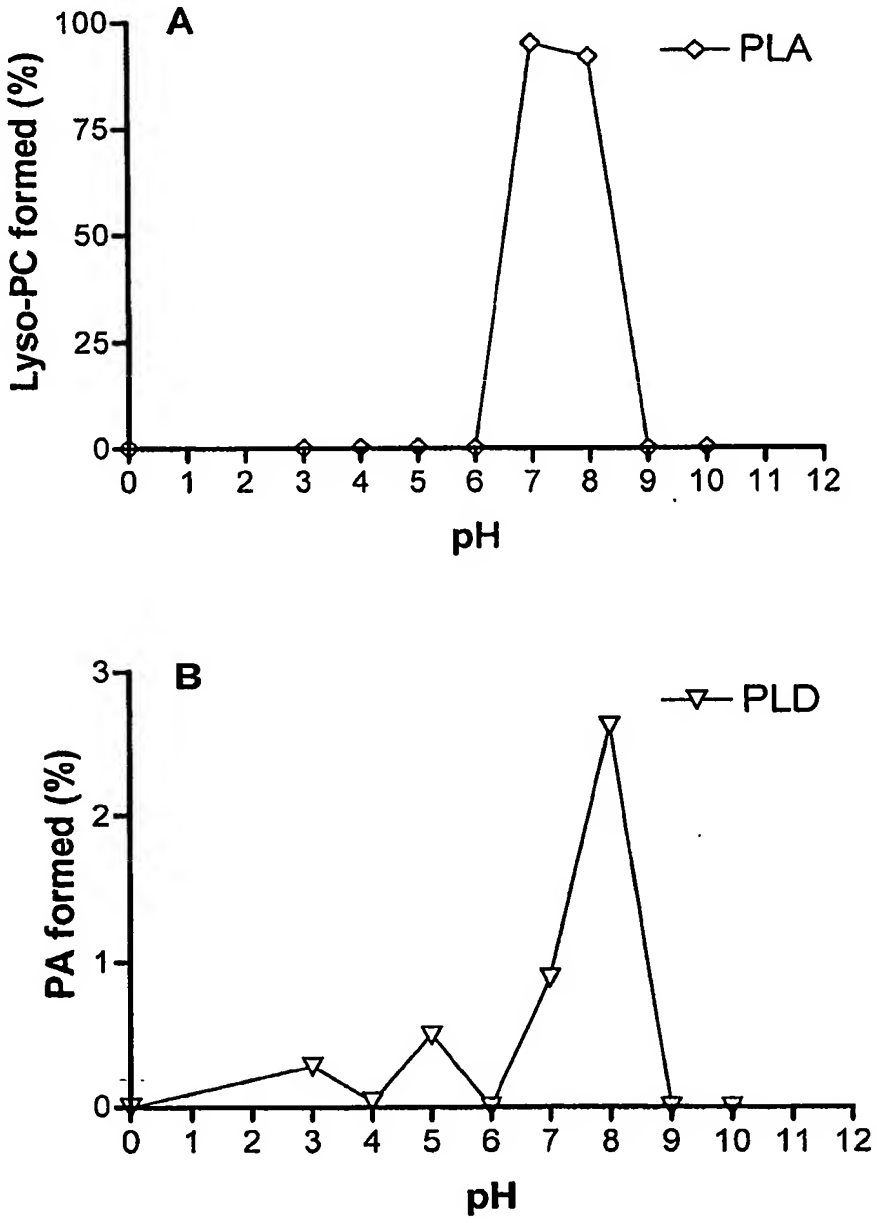

A
PLA
Lyso-PC formed (%)
100
75
50
25
0
0 1 2 3 4 5 6 7 8 9 10 11 12
pH
B
PLD
PA formed (%)
3
2
1
0
0 1 2 3 4 5 6 7 8 9 10 11 12
pH

Figure 6

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/AU04/001570

International filing date: 12 November 2004 (12.11.2004)

Document type: Certified copy of priority document

Document details: Country/Office: AU
Number: 2003906261
Filing date: 13 November 2003 (13.11.2003)

Date of receipt at the International Bureau: 08 February 2005 (08.02.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record.

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.